



Rhythmic Syllable-Related Activity in a Songbird Motor Thalamic Nucleus Necessary for Learned Vocalizations

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Abstract

Birdsong is a complex behavior that exhibits hierarchical organization. It is hypothesized that the hierarchical organization of birdsong is the result of activity in the avian song circuit that selects and activates behavioral units in a specific order. While the representation of singing behavior has been studied in some detail in ‘cortical premotor circuits,’ little is known of the role of the thalamus in the organization of adult birdsong. Using a combination of behavioral and electrophysiological studies, we examined the role of the thalamic nucleus Uvaeformis (Uva) in the production of stereotyped, adult song. Complete bilateral lesions of Uva result in a loss of stereotyped acoustic and temporal structure, similar to earlier reports of the effects of HVC lesions. Notably, Uva lesions result in a broad, nearly exponential distribution of syllable durations, characteristic of early vocal babbling. Using a motorized microdrive, we recorded multiunit activity in Uva during singing in adult birds. We find that neural activity in Uva exhibits significant 10Hz rhythmicity locked to song syllables, increasing prior to syllable onsets and decreasing prior to syllable offsets—a pattern of activity observed in HVC during adult and juvenile song. These results suggest that the avian song is functionally organized around a 10Hz rhythm, with one cycle of the 10Hz rhythm being the fundamental ‘unit’ of song.

List of Abbreviations

Abbreviation

Uva	Nucleus Uvaeformis
HVC	HVC (used as proper name)
EP	Expiratory Pulse
IP	Inspiratory Pulse
GTE	Gesture Trajectory Extrema
RA	Robust Nucleus of Arcopallium
Area X	Area X (used as proper name)
NIf	Nucleus Interface
Av	Nucleus Avalanche
nXIIts	Tracheosyringeal portion of the hypoglossal nucleus
SMA	Supplementary Motor Area
RAm	Nucleus Retroambigualis
PAm	Nucleus Parambigualis
DM	dorsomedial medial nucleus of the intercollicular complex
PAG	Periaqueductal Gray
MMAN	Medial Magnocellular Nucleus of the Anterior Nidopallium
DMP	Dorsomedial Thalamic Nucleus
MHb	Medial Habenula
RVl, IOS, PBvl	Various brainstem respiratory-vocal motor nuclei
cVRG and rVRG	Caudal and rostral ventral respiratory group (respectively)
dph	days post-hatch

The nervous systems of modern animals are strikingly diverse and are able to mediate a vast variety of behaviors. The hydra, which lacks a true brain or muscles, has one of the simplest nervous systems in nature; yet, with only a few hundred neurons, the hydra is able to adapt to noxious stimuli, respond to light and capture prey (Burnett and Diehl 1964). With a few hundred thousand neurons, the honey bee is able to build complex colonies, perform intricate dances and coordinate swarm behavior. The sperm whale's 8kg brain is the largest on the planet and with it the whale is able to navigate vast stretches of the ocean, dive into the darkest depths of the seas and communicate within its intricate social networks using complex vocalizations. Our own brains allow us to share and convey the finest subtleties of human experience and emotion through language, art and music. Indeed, the human brain is the only known organ that seeks to understand itself.

How do a collection of neurons come together to produce the vast array of complex behaviors we observe in the animal kingdom? Beyond satisfying basic human curiosity, addressing this question will prove critical to the development of new therapies for the treatment of many cognitive and movement disorders. The past century has brought immense advances in our understanding and treatment of many common medical problems but the field of neurology lags behind. Movement disorders, such as Parkinson's disease, make up a significant portion of the global burden of disease. One study, based on the Bruneck study cohort, found that the prevalence of all common categories of movement disorders was 28.0% in people ages 50-89 (Wenning et al. 2005). Speech disorders are one of the most common disabilities in the United States and have a profound effect on an individual's interactions within society. Over 1.5 million children ages 3-21 suffer from some communication disorder (Disorders 2010). In addition, over 80,000 patients per year are diagnosed with aphasia, with the majority of these cases due to stroke (Ellis, Dismuke, and Edward 2010).

“Action Syntax” and the sequential organization of motor behavior

Imagine a monkey climbing a tree. As it ascends to the canopy, it pushes off its right leg, extends its right arm forward and flexes its left leg upwards. As his right arm reaches forward, his fingers curl and his right hand grasps a branch. The monkey then pushes off its left leg and

reaches forward with its left arm while simultaneously flexing his right leg. After several renditions of this sequence of movements, the monkey finally reaches the tree top where it can enjoy the warmth of the sun and a well-deserved snack, a ripe banana.

The monkey's act of climbing a tree, as with many complex motor behaviors, consists of multiple single movements linked together in a specific spatial and temporal configuration. Without proper spatial control, the monkey would be unable to navigate its environment: it would reach for the wrong branch, grasp a leaf rather than a sturdy limb and accidentally fall to its death. Equally important and often underappreciated is that the monkey's movements must occur in a specific order; if the monkey moves an arm when he was to move a leg or *vice versa*, he may be unable to climb the tree and may never leave the ground.

This issue of temporal integration is well illustrated by speech and language. Take the sentence "Grandma ate the sandwich." The line conjures up images of a pleasant, older woman sitting in the dining room enjoying an afternoon snack. Rearrange the words and a new sentence is formed: "The sandwich ate Grandma." This line is now reminiscent of a cheesy 80s cult horror film. By changing the order of the words and order by which the sounds are produced by the vocal tract, two sentences of very different meanings are formed. The problem of coordinating multiple, single-component movements into organized sequential temporal patterns have been referred to as the "action syntax" problem (Lashley 1951). Implicit in the idea of the "action syntax" is a hierarchical organization of behavior.

Many complex behaviors observed in nature exhibit a behavioral hierarchy. First described by Lashley (1951), a behavioral hierarchy is a system in which behaviors can be divided into 'units' which themselves can be divided into simpler subunits. It was hypothesized that complex behaviors are formed by neural circuits that select and activate these behavioral units or subunits in a specific order. This hierarchical organization leads to key questions regarding the neural implementation of complex motor behaviors: Are elements of the hierarchy explicitly represented in neural circuits? And if so, how are units or subunits represented and initiated?

Needless to say, all complex motor behaviors require sequential motor control but it is unclear how the brain regulates the sequential order of motor programs in order to form a smooth, cohesive behavior. Through a combination of human and primate studies, some strides have been made in understanding how the brain controls temporal structure of complex movements. In the 1960s, Luria described a series of patients with lesions in the “parasagittal region of the premotor cortex.” These patients had disturbances in organizing movements in correct temporal sequences without problems in single movements or defects in spatial motor control (Luria 1962). A decade later, it was found that patients with ablations of the supplementary motor area (SMA) had a deficiency in performing alternating serial hand movements (Laplante et al. 1977). In monkeys, lesions of the SMA also impair the animals ability to perform sequential motor tasks (Brinkman 1984); similar results were seen with transient chemical inactivation of the SMA in primates (Shima and Tanji 1998). Interestingly, monkeys were still able to perform sequential motor tasks when prompted by sensory cues. However, this ability was also lost when premotor areas were lesioned (Halsband and Passingham 1982).

Recordings in the SMA and pre-SMA in behaving monkeys have demonstrated that many neurons in these regions are active during movement and that their properties differ from those of neurons in the primary motor area under the same conditions. In particular, activity of neurons in the SMA and pre-SMA is strongly associated with the temporal sequence of motor behaviors and not the individual movements themselves or their spatial control (Mushiake, Inase, and Tanji 1990; Matsuzaka and Tanji 1996). These results, together with clinical observations in stroke patients, suggest that the SMA and pre-SMA play a critical role in generating and controlling sequential motor behavior.

The SMA, pre-SMA and premotor area form extensive connections with various subcortical motor areas including the basal ganglia, cerebellum and thalamus; given the extensive involvement of these cortical motor areas in performing sequential motor tasks, it is reasonable to postulate that the subcortical areas intimately connected to them are also involved. Indeed, impairments in sequential motor behaviors have been documented in patients with Parkinson’s disease (Benecke et al. 1987; Harrington and Haaland 1991) and Huntington’s disease (Thompson et al. 1988). In primates, pharmacological inactivation of the dorsal striatum

leads to deficits in performance (Van Den Bercken and Cools 1982; Miyachi et al. 1997) and learning (Miyachi et al. 1997) of sequenced movements. Recordings of pallidal neurons in monkeys while they performed a tracking sequential task revealed neurons with phase-specific activity that was sequence dependent, consistent with the basal ganglia having a role in ordering of movements (Mushiake and Strick 1995). With respects to the cerebellum, muscimol injections into the cerebellar nuclei do not inhibit the learning of sequential behaviors; however, injections into the dentate nucleus do slow down movements and increase the number of errors in performing learned motor sequences (Lu, Hikosaka, and Miyachi 1998).

In mammals, the cortex, basal ganglia, cerebellum and brainstem all play key roles in the planning and production of complex motor behaviors. Of note, all of these motor areas send projections to the motor thalamus. However, despite its central location in the motor system, little is understood of the role of motor thalamus in the production of complex learned behaviors, particularly in determining temporal structure of the complex movements. However, limited studies in humans do suggest that the thalamus does play some role in organizing sequential behaviors. MacMillan (2004) performed microelectrode recordings in patients undergoing stereotactic neurosurgery for implanting deep-brain-stimulating (DBS) electrodes in thalamus. Using a sequential button press task, thalamic neurons were identified with preparatory, delay-period, task and phase-specific activity (MacMillan et al. 2004). While this work supports a role for the motor thalamus in performing multiple movements in a correct temporal order, more work is necessary in order to determine how thalamic output is ultimately translated to a motor sequence and how activity of the thalamus, cortex, basal ganglia and cerebellum are ultimately coordinated and integrated.

Birdsong and singing behavior

The songbird has emerged as a fantastic model system for the production and learning of complex motor behaviors, such as speech. Over the last few decades, neuroscientists have acquired an increasing knowledge of the neural circuitry that mediates singing behavior. Recent work in comparative neuroanatomy has challenged the old notion that the avian telencephalon consists mostly of basal ganglia with very few cortical regions (Erich D Jarvis et al., 2013, E D Jarvis et al., 2011). Indeed, large portions of the avian forebrain, including areas used for song

behavior, are analogous to the mammalian neocortex. In addition, songbirds possess basal-ganglia circuits with striking homology to those in mammals (Doupe, Perkel, Reiner, & Stern, 2005; Goldberg & Fee, 2010; Person, Gale, Farries, & Perkel, 2008). The avian “cortex,” unlike the mammalian neocortex, conserves the nuclear organization characteristic of subcortical regions. As a result, the song subcircuit is well-defined, which makes experimental manipulation easier (Michale S Fee and Scharff 2010). In addition, the development of methods to allow for the recording of all vocalizations of a male zebra finch has made detailed quantification of the song learning process possible (Tchernichovski et al. 2001).

Birdsong, like many complex motor tasks, is thought to exhibit a hierarchical behavioral organization. Adult zebra finch song consists of a stereotyped sequence of 3-5 song syllables, which together form a repeated song motif of about 0.5-1 second duration. During a single bout of singing, the motif may be repeated multiple times. In addition, bouts of singing are often preceded by a series of short, soft vocalizations called introductory notes (Zann 1996). It is unclear how this behavioral hierarchy is represented in vocal premotor and learning circuits of the avian brain.

These behavioral and physiological observations strongly suggest that the syllable forms the fundamental ‘unit’ of song production. This conclusion leads to a key question regarding the neural basis of vocal behaviors: Is the modular organization of song reflected in its underlying neural representation in the avian song motor system?

The avian song circuit: a bird’s eye view

Over the last few decades, the neural circuitry underlying the production of adult song has been well described. The avian vocal network that generates song can be viewed as a combination of a feed-forward premotor pathway combined with a feedback pathway (Figure 1). The feedforward pathway includes HVC (used as a proper name), a premotor nucleus in the avian pallium (Nottebohm, Stokes, and Leonard 1976; Bottjer et al. 1989; Vu, Mazurek, and Kuo 1994), an analog of the mammalian neocortex (Karten 1991; Erich D Jarvis 2007). HVC projects to the robust nucleus of the arcopallium (RA) (Nottebohm, Stokes, and Leonard 1976), an avian homologue of layer V primary motor cortex (E D Jarvis et al. 2011; Reiner et al. 2004). Neurons

in HVC that project to RA generate a sparse, stereotyped sequence of bursts throughout the motif (Hahnloser, Kozhevnikov, and Fee 2002; Kozhevnikov and Fee 2007; Long, Jin, and Fee 2010). RA neurons, which generate complex sequences of bursts during singing (Hahnloser, Kozhevnikov, and Fee 2002; Leonardo and Fee 2005), provide inputs to motor neurons in the hypoglossal nucleus that innervate muscles of the vocal organ (Vicario and Nottebohm 1988). Neurons in RA also project to brainstem respiratory nuclei RAm (nucleus retroambigualis) and PAm (nucleus parambigualis) (Reinke and Wild 1997; Vicario 1991; Reinke and Wild 1998; Kubke et al. 2005). RA also innervates the midbrain vocalization center DM (dorsomedial medial nucleus of the intercollicular complex), an area thought to be analogous to vocal centers in the mammalian periaqueductal gray (Dubbeldam and den Boer-Visser 2002).

Nearly all of the brainstem and midbrain circuits that receive axonal inputs from RA, in turn send a projection back to HVC through the higher-order thalamic nucleus Uvaeformis (Uva), forming an anatomical brainstem-thalamocortical loop (Ashmore, Renk, and Schmidt 2008; Ashmore, Wild, and Schmidt 2005; Nottebohm, Kelley, and Paton 1982; Striedter and Vu 1998b). As well as its brainstem motor projections, Uva receives multiple somatosensory, auditory and visual inputs and is innervated by cholinergic fibers (M J Coleman et al. 2007; J M Wild 1994; Akutagawa and Konishi 2005). In addition to HVC, Uva also sends inputs to two other forebrain nuclei in the efferent pathway: NIf and Avalanche (Av) (Akutagawa and Konishi 2010). Here, we will briefly describe some recent studies on the mechanism by which these motor-associated nuclei produce the complex motor commands underlying adult song.

HVC

Nucleus HVC is a forebrain song nucleus necessary for the production of adult, stereotyped song (Aronov, Andalman, and Fee 2008; Nottebohm, Stokes, and Leonard 1976). Based on genetic expression profiles and cell morphology, HVC is similar to layer III of the mammalian premotor cortex (Erich D Jarvis et al. 2013). HVC receives projections from at least four other nuclei in the avian brain: the cortical nuclei NIf, Avalanche and MMAN, and the thalamic motor nucleus Uvaeformis (Uva) (Nottebohm, 2004; Akutagawa & Konishi, 2010; Foster, Mehta, & Bottjer, 1997). In turn, HVC projects to three cortical nuclei: RA, Area X (the avian basal ganglia, important in song learning) and Avalanche (Nottebohm and Arnold 1976;

Bottjer et al. 1989; Akutagawa and Konishi 2010). HVC neurons that project to RA and Area X form distinct populations and therefore are called *HVC_{RA} neurons* and *HVC_X neurons*, respectively. HVC also contains at least one class of local interneurons, referred to as *HVC_I neurons* (Mooney and Prather 2005).

HVC is necessary for mature singing in adult birds. Complete bilateral lesions of HVC profoundly alter adult singing behavior, but have differential effects on directed and undirected song. First, HVC lesions completely abolish directed song. Specifically, when presented with a female, HVC-lesioned birds approach the female and appear to attempt singing, but no vocalizations are produced. In contrast, birds with HVC lesions can still produce song in social isolation (undirected song), but their vocalizations lack the stereotyped structure of normal adult song. In fact, the undirected song produced by HVC-lesioned birds is highly variable and resembles subsong, the most juvenile ‘babbling’ form of singing (Nottebohm, Stokes, and Leonard 1976; Aronov, Andalman, and Fee 2008; Andalman and Fee 2009; Olveczky, Andalman, and Fee 2005).

It has been shown that HVC plays a critical role in the timing of song at all temporal scales. Local, bilateral cooling of HVC slows the temporal structure of song at all timescales including a lengthening of both acoustic and respiratory patterns. In contrast, local cooling in RA, a motor nucleus downstream of HVC, has no effect (Long and Fee 2008; Andalman, Foerster, and Fee 2011). Electrophysiological recordings in HVC reveal that HVC projector neurons generate highly sparse bursts of activity, with different neurons active at different times in the song (Hahnloser, Kozhevnikov, and Fee 2002; Kozhevnikov and Fee 2007; Long, Jin, and Fee 2010).

The observations of sparse bursting in HVC have inspired several models of HVC coding dynamics. In one model, bursts of activity in HVC only occur at discrete times in the song corresponding to specific events in the song. These events, collectively referred to as gesture trajectory extrema (GTEs), include syllable onsets, offsets and extrema of gestures (Amador et al. 2013). Notably, in the GTE model, HVC does not play a premotor role in controlling these vocal gestures, but rather receives an efference copy of vocal-respiratory motor output initiated in the brainstem (Alonso et al. 2015).

A different model of HVC function hypothesizes that HVC bursts occur continuously throughout song vocalizations, and that these bursts play a direct premotor role in song production. According to this hypothesis, nearly all temporal features of the song are encoded in the sparse bursting of projecting neurons in HVC. A distinct, but related part of this model suggests that sequential bursting in HVC is mediated by multiple, synaptically-connected chains (Long, Jin, and Fee 2010). In this model, each chain controls the timing of a single syllable and the chains are activated by the midbrain-thalamic feedback loop, which serves not only to initiate activity in the chain, but to synchronize this initiation across hemispheres (Marc F Schmidt 2003; Andalman, Foerster, and Fee 2011; Michale S. Fee and Long 2013).

Robust Nucleus of the Arcopallium (RA)

As previously mentioned, RA is an avian homologue of layer V primary motor cortex (Erich D Jarvis 2004). RA innervates several brainstem respiratory and vocal control nuclei as well as the midbrain nucleus DM and the thalamic nucleus DLM (Vicario 1991; Vates, Vicario, and Nottebohm 1997). In male birds, RA consists of approximately 16,000 neurons with approximately 8000 RA neurons projecting to the brainstem (Gurney 1981). These RA projectors control the activity of seven syringeal muscles (Crawford H. Greenewalt 1968), with approximately 1:1000 convergence of neurons to muscles.

RA is critical in the production of song; bilateral lesions of RA entirely block singing in juvenile and adult birds (Aronov, Andalman, and Fee 2008; Nottebohm, Stokes, and Leonard 1976). Electrophysiological studies reveal that, during singing, RA neurons generate a complex and stereotyped sequence of high frequency bursts of spikes. Each RA neuron, on average produces roughly 12 bursts per song motif, each ~10ms in duration. As a population, RA neurons are active throughout the song vocalization, with 12% of RA neurons active at any point in time. Theoretically, there are two mechanisms by which a bird can produce the same sound within the song motif: 1) by reactivating the same RA ensemble to produce the same sound or 2) activating a degenerate RA sequence consisting of a unique combination of neurons. Surprisingly, it appears that both mechanisms are utilized by the bird to produce repeating elements in the song (Leonardo and Fee 2005).

These results, together with the studies done in HVC, suggest that zebra finch song is driven by a dynamic circuit that operates under on a single underlying clock, HVC. The sparse code generated by sparsely bursting HVC_{RA} neurons drives neurons in RA. Ultimately, through the large convergence of RA neurons to vocal control muscles (resulting in a many-to-one mapping of RA activity to song structure), the sparse representation of the song observed in HVC is transformed into the continuous activity of the vocal muscles (Michale S Fee, Kozhevnikov, and Hahnloser 2004).

Brainstem respiratory and vocal centers

In songbirds, vocalizations are generated by passing air through the syrinx. The songbird syrinx is a bipartite vocal organ, with each half controlled independently by two sets of seven syringeal muscles on each side. Unlike human laryngeal muscles, which are largely controlled by the vagus nerve, the avian syringeal muscles are innervated by the tracheosyringeal portion of the hypoglossal nucleus (nXIIts) (Paton, Manogue, and Nottebohm 1981; Vicario and Nottebohm 1988). Vocalization also requires major adjustments in respiration; the changes in pressure and airflow necessary to produce vocalizations are mediated by the spinal motor neurons which innervate the ventilator muscles (Suthers and Zollinger 2004). Ultimately, for proper vocalization, birdsong requires precise coordination between these output pathways in order to produce appropriate vocalizations.

While majority of birdsong studies have focused on the telencephalic structures and pathways responsible for high level patterning of song (e.g. HVC and RA), little attention has been given to motor nuclei located in the brainstem. The brainstem respiratory-vocal network consists of a number of neuronal centers include the midbrain structure DM, the respiratory control nuclei PBvl, IOS, RVL, PAm and RAm, and the vocal motor nucleus of the hypoglossal nerve (nXIIts) (Vicario and Nottebohm 1988; Reinke and Wild 1997; Reinke and Wild 1998; J. M. Wild 2004). Each of these areas receive inputs from RA: middle and ventral RA neurons project topographically onto nXIIts motor neurons that innervate either dorsal or ventral syringeal muscles, while dorsal RA neurons largely project onto the respiratory control nuclei located in the lateral medulla (Vicario 1991; J Martin Wild 1993; Reinke and Wild 1998). It has been assumed

that RA plays the largest role in coordinating respiratory and vocal motor control. However, the rich connections within the brainstem vocal-respiratory network suggest that much of the processes involved in coordinating respiratory and vocal motor control may occur within the brainstem itself (J. M. Wild 2004).

Anatomical studies suggest that the neural network formed by avian RAm and PAm may serve as a nexus for coordinating respiratory and vocal motor control during singing. The respiratory nucleus RAm and PAm are thought to be the avian analogue of the mammalian caudal and rostral ventral respiratory groups (cVRG and rVRG), respectively, which serves as the final common pathway for vocalizations (Reinke and Wild 1997). In mammals, the VRG receives inputs from periaqueductal gray (PAG) and the pontine call site, brainstem nuclei thought to mediate basic, innate vocalizations in a wide variety of animals (Kittelberger 2006; Larson 1985; Subramanian, Balnave, and Holstege 2008; Gerrits and Holstege 1996). Similarly, in birds, in addition to descending projections from RA, RAm also receives descending projections from the midbrain, specifically from the avian analogue of PAG, the dorsomedial nucleus of the intercollicular complex (DM) (Dubbeldam and den Boer-Visser 2002). In turn, RAm and PAm send projections to the spinal motor neurons innervating expiratory and inspiratory muscles, respectively, as well as XIIIts (J. M. Wild 2004). Retrograde labeling studies have shown that the population of RAm neurons innervating motor neurons in XIIIts are anatomically distinct from those innervating motor neurons in the thoracic spine that control respiration (J. M. Wild 2004; Sturdy, Wild, and Mooney 2003). Labeling studies have also revealed two other populations of neurons in RAm. The third population consists of large, multipolar, vagal neurons which have unknown targets. Finally, there is a fourth population of ascending neurons which synapse onto PAm, RVl, IOS, PBvl and DM (J. M. Wild 2004).

Several electrophysiology studies have been performed in order to better define the properties of RAm to XIIIts projector neurons. *In vitro* and *in vivo* intracellular recordings XIIIts performed in adult male zebra finches revealed that RAm provides both excitatory and inhibitory inputs to the syringeal motor nucleus (Sturdy, Wild, and Mooney 2003). In a separate study, intracellular recordings in a zebra finch brain stem slice preparation identified two distinct populations of RAm projector neurons: type I and II. Little is known regarding the function of type

I projectors. On the other hand, type II projectors are inhibitory neurons that project bilaterally to XIIIts and likely represent the inhibitory inputs previously described by *in vivo* intracellular recordings (Kubke et al. 2005). Interestingly, type II neurons can exhibit either bursting or non-bursting activity, depending on how hyperpolarized the membrane of the neuron is prior to excitation. While the actual mechanism is still unclear, type II projectors may play a key role in coordinating vocal motor and respiratory patterns during song.

Ascending avian song circuit: Nucleus Uvaeformis (Uva)

Nucleus Uvaeformis (Uva) is a motor thalamic nucleus approximately 250µm in diameter. Uva receives bilateral inputs from two nuclei in the brainstem vocal-respiratory network: DM and PAm (Striedter and Vu 1998a; Reinke and Wild 1998). Uva also receives inputs from the visual, somatosensory and auditory systems, as well as cholinergic inputs from the medial habenula (MHb) (J M Wild 1994; Akutagawa and Konishi 2005; M J Coleman et al. 2007). In turn, Uva sends outputs to three cortical nuclei: HVC, Nif and Avalanche (Nottebohm, Kelley, and Paton 1982; Akutagawa and Konishi 2010). While the role of Uva in adult song production is unclear, several possible functions have been proposed. Because it is part of a bilaterally connected ascending pathway, it has been suggested that Uva plays a critical role in synchronizing activity in the two hemispheres of the telencephalon during singing (Marc F Schmidt 2003; Gibb, Gentner, and Abarbanel 2009). Uva may also play a role in determining the order of syllables during singing (Gibb, Gentner, and Abarbanel 2009). It has also been hypothesized that Uva may play a role in linking syllable-length chains such that the end of one syllable chain activates the beginning of the next syllable chain through this feedback loop (Michale S. Fee and Long 2013). In contrast to the model in which the entire motif is generated by a single chain in HVC, in this latter view, Uva could play a central role in selecting or activating song syllables.

Bilateral synchronization of forebrain activity

The syrinx, the avian vocal organ, is a bipartite structure, with each half capable of functioning independently in sound generation. Because each half of the syrinx can be independently controlled to produce vocalizations, exquisite coordination is required between the syringeal motor commands that control each side (Goller & Suthers, 1996; J M Wild et al., 2000). However, several features of the avian song system make coordination a particularly challenging

task. Unlike in the human language circuit, the avian song system is bilaterally organized and is anatomically symmetric across hemispheres (Paton, Manogue, and Nottebohm 1981). Each half of the syrinx is controlled by inputs from song nuclei on the ipsilateral side (J Martin Wild 1997), with the motor commands that control the system ultimately originating from the forebrain (Nottebohm, Kelley, and Paton 1982; Vicario 1991). To complicate matters further, the bird brain lacks a corpus callosum, preventing communication across hemispheres at the level of the forebrain (M F Schmidt and Ashmore 2008). In theory, without any mechanism to maintain coordination across hemispheres, any small perturbation in activity in one hemisphere can result in a temporal misalignment of motor commands and result in abnormal vocalizations.

Given the lack of connections between hemispheres at the level of the forebrain, it has been suggested that subcortical structures may play a key role in synchronizing premotor activity across hemispheres during song production. There are at least three different anatomical pathways that could serve the function of synchronizing premotor activity in the song network. All three pathways originate in RA and project back to HVC via the thalamus. The three pathways are 1) $RA \rightarrow DM \rightarrow Uva \rightarrow (NIf) \rightarrow HVC$ 2) $RA \rightarrow PAm \rightarrow Uva \rightarrow (NIf) \rightarrow HVC$ and 3) $RA \rightarrow DMP \rightarrow MMAN \rightarrow HVC$ (J M Wild, Williams, and Suthers 2000; Gibb, Gentner, and Abarbanel 2009). Of these three pathways, the last pathway involving MMAN is considered least likely to play a critical role in synchronizing premotor activity. Lesions of MMAN in adult birds have a minor effect on singing mainly restricted to introductory notes and suggesting MMAN is unlikely to be necessary in the synchronization of premotor activity (Foster and Bottjer 2001). This result would suggest that feedback circuits involving Uva play a critical role in maintaining synchrony across hemispheres during singing.

Coordination of respiration and vocalization

Many models of complex motor control, like those of song production, are framed in a hierarchical and linear manner and highlight a system of “top-down” control. In these models, signals from the forebrain drive the activity of brainstem nuclei, either by selecting and initiating innate motor programs or by completely overriding these pre-existing circuits (Jürgens 2002; Krauzlis 2004). Despite the intuitive appeal of the “top-down” model, it argues that the brainstem plays only a passive role in the patterning of complex behaviors. However, these models do not

account for the role brainstem-cortical feedback connections play in the patterning of sequential motor programs. Indeed, a growing body of evidence suggest that ascending connections from the brainstem to the cortex play a critical role in patterning complex motor behaviors (Sommer and Wurtz 2004a; Sommer and Wurtz 2004b; Sommer and Wurtz 2008; Wurtz, Sommer, and Cavanaugh 2005; Poulet and Hedwig 2006; Pynn and DeSouza 2013; Blakemore, Wolpert, and Frith 2000).

In both humans and songbirds, vocalization requires the precise coordination of respiratory and vocal-motor centers (Riede and Goller 2010; Levelt 1993). In adult birds, song syllables are produced during pulses of positive air sac pressure (expiratory pulse) while silent gaps are associated with pulses of negative air sac pressure (inspiratory pulses) (Franz and Goller 2002). This remarkably tight coordination between respiration and vocalization has led to the suggestion that forebrain circuits, particularly the premotor nucleus HVC, play a critical role in coordinating these motor processes (J Martin Wild 1998). Indeed, manipulations of HVC activity during singing affect both patterns of vocalization and respiration. Localized cooling of HVC has been shown to slows the temporal structure of song at all timescales (Long and Fee 2008), including the fine acoustic structure within syllables, and the duration of syllables and gaps. Cooling of HVC also increases the duration of both expiratory pulses (EPs) and inspiratory pulses (IPs). Interesting, while cooling uniformly stretches EPs, cooling appears to stretch most IPs non-uniformly (Andalman, Foerster, and Fee 2011). This suggests that while the expiratory phase is directly driven by the nucleus HVC, the inspiratory phase is primarily driven by autonomous respiratory networks within the brainstem (Andalman, Foerster, and Fee 2011).

It has been proposed that HVC is composed of discrete chains of synaptically connected neurons, with each chain related to a particular syllable (Long, Jin, and Fee 2010). Given findings from the HVC cooling experiments, it has been proposed that each chain controls the moment-to-moment timing during expiration. The end of each chain initiates an inspiratory pulse (IP). During the IP, the inspiratory respiratory centers are thought to reactivate HVC via Uva. The IP is then terminated by the next HVC chain at the onset of the next syllable (Andalman, Foerster, and Fee 2011). A clear prediction of this hypothesis is that Uva should carry syllable-onset-related signals.

Previous studies

While the role of forebrain nuclei in the production of stereotyped adult song has been well described, surprisingly little is known of the role of Uva in adult birds during vocalization. Previous studies have found that partial lesions of Uva in adult birds lead to disruption of normal, directed song. Partial unilateral or bilateral Uva lesions disrupted the stereotyped order of syllables within a motif. While the acoustic structure of syllables remained intact, partial Uva lesions increased variability in syllable order (Williams and Vicario 1993). More complete lesions of Uva have an even more pronounced effect on directed song. Uva lesions transformed the normally stereotyped sequences of syllables into long trains of repeated introductory notes that never transition to an ordered series of song syllables (Melissa J Coleman and Vu 2005). However, these earlier lesion studies did not address the difference between directed and undirected song in the context of lesions in the motor pathway that was later discovered. (Aronov, Andalman, and Fee 2008). It has been established that the effects of lesions on the motor pathway can vary depending on the context in which birds are singing. For example, HVC lesioned birds do not sing when presented with a female (directed song) but do sing when placed in social isolation (undirected song) (Aronov, Andalman, and Fee 2008).

Previous electrophysiology studies of Uva reported premotor bursts that preceded calls and introductory notes by 50-90 ms and increased activity during song motifs (Williams and Vicario 1993). This study also reported elevated activity, referred to as “super bursts,” locked to the offsets of song motifs. To further examine the electrophysiological properties of Uva neurons during singing, we have taken advantage of the technique of antidromic stimulation to specifically target neurons in the core of Uva that project to HVC. This approach is advantageous because Uva is a very small, deep nucleus (~250µm across and 5.2mm below the brain surface) and thus is difficult to target.

Strategies

Here we address, using a combination of lesions and electrophysiology recordings, the role of Uva in adult song. It has been hypothesized that these recurrent connections serve to relay brainstem activity during singing back to the forebrain nuclei NIf and HVC. Specifically, we

hypothesized that ascending connections from Uva relays an efference copy of brainstem motor output back to the premotor nucleus HVC. We hypothesize that these signals from the brainstem play a critical role in determining the temporal structure of song and serves to activate HVC prior to syllable onsets as well as synchronize premotor activity across hemispheres. We find that, similar to lesions of HVC, bilateral Uva lesions abolish adult stereotyped song. Unlike adult song, the post-lesion songs exhibit no distinct identifiable syllables and have a broad, nearly exponential distribution of syllable durations, which is characteristic of subsong, the most juvenile form of birdsong. Multiunit recordings in Uva revealed elevated activity during song with a distinct pattern of activation prior to syllable onsets and dips prior to syllable offsets. These modulations are strongly correlated with song amplitude and are coherent with a pronounced 10Hz rhythmicity in song structure. Altogether, our findings suggest that activity in Uva is strongly related to syllable onsets and offsets, and in particular with the rhythmic component of these events. We find no evidence for a specific representation in Uva of other aspects of the song hierarchy, including song motifs or song bouts.

Materials and Methods

Subjects were adult male zebra finches, >90 days post hatch (dph). Birds were obtained from either the Massachusetts Institute of Technology breeding facility or a commercial breeder. Animal care and experiments were performed in accordance with the National Institute of Health guidelines and approved by the Massachusetts Institute of Technology Institutional Animal Care and Use Committee.

Sound recordings: Several days prior to surgery, birds were placed in custom-made sound isolation chambers. Vocalizations were recorded with custom-written Matlab software or with Sound Analysis Pro, which were configured to record the soft vocalizations of subsong.

Antidromic Identification of Uva: Uva was localized by antidromic stimulation from HVC. Before surgery, anesthesia was induced with 1-3% isoflurane in oxygen. After mapping out HVC as described below, a bipolar stimulating electrode was implanted in HVC for antidromic identification of Uva. Single monopolar pulses of 0.2ms duration was produced using an isolated stimulation unit (AMPI, Inc) controlled by a Master 8 (AMPI, Inc), with intensities varying from 50-200 μ A. Uva neurons were found using ongoing 1 Hz stimulation in HVC to elicit spike responses.

Localization of HVC: We localized HVC by antidromic stimulation of Area X. A bipolar stimulating electrode was implanted into Area X using stereotaxic coordinates (Head Angle: 0°, AP: 5.40, ML:1.50, DV:-2.80). HVC neurons were identified by an ongoing 1Hz monopolar pulse of 0.2ms duration, with intensities varying from 50-200 μ A. After localizing HVC, a retrograde tracer (dextran) was injected into HVC in order to label HVC-projecting Uva neurons.

Lesions: The location of Uva was identified and mapped by antidromic stimulation in HVC. After Uva was located, electrolytic lesions were made using a 1M Ω Pt-Ir electrode (MicroProbes, PI20031.0A3). To ensure a complete lesion, approximately -20 μ A of current was passed for 60 s, usually at two locations 150 μ m apart along the anterior-posterior axis. Prior to implantation of the stimulating electrode in HVC, a retrograde neuronal tracer (dextran) was injected into HVC bilaterally to permit later assessment of the extent of Uva lesion. After

surgery, the birds were allowed to recover from surgery and then placed back into the sound isolation chambers. Birds typically began to sing again 1-3 days post-surgery. Both directed and undirected song was recorded for three days beginning from the first day of singing post-surgery.

Chronic Neural Recordings in Uva: Experiments were carried out using a motorized microdrive as previously described (M S Fee and Leonardo 2001; Okubo, Mackevicius, and Fee 2014). The microdrive weighed ~1.5g and contained a single microelectrode (MicroProbes, PI20035.0A3). As the bird sang, the electrode was advanced slowly throughout the dorsal-ventral extent of Uva. A small lateral positioner allowed us to displace the electrode by several tens of micrometers in order to make a fresh penetration through Uva. The HVC-projecting neurons of Uva were identified by antidromic stimulation via HVC. We were able to record single units in Uva under anaesthesia and in awake, non-singing birds. However, we found that during singing, Uva neurons spiked at very high rates making single unit isolation impossible. On the final day of recording, the recording electrode was retracted ~200µm above Uva and an electrolytic lesion was made through the recording electrode (-15µA for 15sec) allowing histological confirmation of the placement of the electrode tip.

Histology: Following the last day of recordings, birds were given an overdose of sodium pentobarbital and perfused transcardially with 0.2 M phosphate-buffered solution followed by 4% paraformaldehyde in phosphate-buffered solution. Brains were post-fixed overnight and cut into 100 µm thick sagittal sections on a vibratome. Sections were stained for the neuronal marker NeuN (Millipore, A60) and mounted. Uva lesions were confirmed by the absence of retrogradely-labeled HVC-projecting cells in the thalamus. Proper injection of retrograde tracer into HVC was confirmed by the presence of retrogradely labeled cells in nucleus interface (NIf).

Data Analysis

Sound Analysis: All data analyses were performed with custom MATLAB software. Syllables and gaps were segmented based on the analysis described by Aronov et al., 2011. The audio signal was preprocessed with a 1-4 kHz bandpass filter. The sound amplitude was determined by squaring the audio signal and smoothing it with a 2.5ms (SD) Gaussian function. The relative sound level was converted to decibels by taking the logarithm (base 10) of the processed audio

signal and multiplying it by 10. Sound amplitude produced during singing is bimodally distributed, corresponding to vocalized syllables and silent gaps. The mean and SDs of these two mode were estimated by fitting two Gaussian curves to the sound level distribution using expectation maximization.

For syllable segmentation in each recording, we calculated a sound threshold as the Fisher discriminant of two Gaussian modes (corresponding to noise and sound) fit to the values of log-amplitude. We detected crossings of this threshold and defined sound onsets and offsets as the closest points to these crossings where amplitude deviated from noise by 2 standard deviations. Sounds separated by <7 ms of silence were merged into a single syllable, and segments of sound <7 ms long were eliminated. Bouts were defined as a sequence of syllables with gaps no longer than 300ms. Syllable renditions with noise or female calls were removed from the analysis. All syllable onsets and offsets were manually verified for accuracy.

To quantify the extent to which Uva-lesioned song resembles subsong, we carried out an analysis of the distribution of syllable durations (Veit, Aronov, and Fee 2011). Syllables and gaps were initially analyzed by fitting an exponential function to their duration distribution using maximum-likelihood estimation (MLE). This analysis was performed on song data collected during one day of singing, and consisted of 1000-10,000 syllables. The goodness-of-fit (Γ) of the exponential was estimated using the Lilliefors statistics (Lilliefors, 1969). Distributions that are similar to subsong and are well fit by the exponentials typically have a goodness-of-fit metric <2 . Distributions similar to early plastic song and are beginning to exhibit a protosyllable peak, typically have values >2 .

Song Rhythmicity: Song rhythmicity was determined according to the analysis described by Saar et al., 2008. To compute song rhythm, we first extracted the sound amplitude during song bouts. Bouts were defined as continuous stream of syllables with gaps no more than 350ms. The sound amplitude within each bout was mean-subtracted and the spectral analysis of the song amplitude was computed with the FFT function in Matlab (1 tapers, 2 time half-bandwidth product). The frequency spectrum was then normalized by bout length and squared to obtain the power spectrum. Song rhythmicity was quantified as the height of the largest peak of the ratio between

the normalized power spectrum and the null power spectrum at frequencies greater than 3 Hz. In this case, the null power spectrum was an exponential power spectrum distribution, which is seen in subsong. Only peaks above 3 Hz were considered because these correspond to the typical frequency at which syllables occur during singing (Saar and Mitra 2008).

Maturity Index: To quantify the level of stereotypy, we used the analysis described by Aronov et al., 2008 based on a spectral correlation of different bouts produced by the same bird. Adult song is highly stereotyped and thus exhibits a high degree of spectral correlation across renditions. In contrast, young birds exhibit much less stereotyped song and exhibit a lower degree of correlation across song bouts. Approximately 100 bouts were randomly selected from the data. We only considered bouts that were, at least, 700 ms long and at most 2 s long. These bouts thus included at least two song motifs. Spectrograms were calculated using the multi-taper method (2 tapers, 10 ms window, 1 ms step size, bandwidth parameter of 1.5); (Tchernichovski et al. 2000). For all possible distinct pairs of bouts in this data set, a correlation matrix was calculated by computing the correlation of power spectra between 860 Hz and 8.6 kHz for each pair of 1 ms time slices of the spectrogram. We then measured the maximum value of the lag correlation function. The resulting value was averaged over the ~10,000 comparisons.

Analysis of Neural Activity: Digitized neural activity waveforms were rectified, smoothed with a 2 or 5 ms (SD) Gaussian function.

Time warping for song alignment: The duration of song motifs of zebra finch song can vary from bout to bout by up to 9ms. (Olveczky, Andalman, and Fee 2005). This jitter can introduce considerable noise to the structure of multiunit activity in Uva if each bout is aligned only to song motif onset. To display the neural activity in Uva aligned to a single song motif, we time warped the multiunit activity using syllable onsets and offsets in the motif as alignment points (Leonardo 2004). Digitized neural activity waveforms were rectified, smoothed with a 2 or 5 ms (SD) Gaussian function. Multiunit activity between each alignment point was then either stretched or compressed to match the corresponding interval in a representative template motif. To select the representative template motif, we determined the median motif length and chose

the bout whose length is closest to that value. This piecewise linear time warping was based on the song structure and was independent of the multiunit activity.

Gesture Trajectory Extrema (GTE) analysis: GTE times were extracted from the songs using a previously published automated method (Boari et al. 2015) . The approach is to use a dynamical model of the vocal organ (the syrinx) to infer the trajectory of two control parameters —air sac pressure and labial tension (Amador et al. 2013; Perl et al. 2011). Continuous segments of control parameters are called ‘gestures’, and local maxima in either of the two control parameters within a gesture are called extrema. These, together with the beginning and end of the gesture, are identified as gesture trajectory extrema, or GTEs.

To calculate the null distribution for the cross-correlation between Uva activity and GTE times, the total number of GTEs was redistributed probabilistically across syllables based on syllable length. After redistributing GTEs among the different syllables, the GTEs were redistributed within syllables randomly. GTEs occurring at syllable onset and offset were kept at their calculated times based on the previously described algorithm.

Relationship between Uva activity and vocal output. We analyzed the relationship between Uva activity and vocal output using two methods. First, we calculated the cross-correlation between the Uva multiunit activity and the sound amplitude. We estimated the premotor lag based on at what time lag a peak in the cross-correlogram was observed. We also estimated the covariance between temporal variability in Uva activity and vocal output over a range of time lags using an analysis described by *Ali et al.* Multiple renditions of the song were aligned to an average template (as described above). The warping paths from these alignments were then applied to the corresponding neural traces at various time lags (range: -90 ms to +30ms; negative shifts imply that Uva activity precedes vocalization). For each lag, we calculated the correlation coefficient for all possible neural trace pairs in the block. For each bird ($n = 5$), the procedure was repeated at each recording site; the mean correlation coefficient calculated at each lag time, averaged across blocks, and then converted to a z-score. We then averaged these z-scores across birds to generate a mean ‘covariance’ profile. The latency was estimated by fitting a Gaussian function to the data over a selected range (-20 to -40ms).

Rhythmicity: The spectral analysis of the song amplitude and multiunit activity was carried using code from the Chronux package (Mitra and Bokil 2007, <http://chronux.org/>). Quantities calculated include power spectral density, cross power spectral density, and coherency (1 tapers, 2 time half-bandwidth product). Digitized neural activity waveforms were rectified, smoothed with a 2ms (SD) Gaussian function. The null distributions for coherency and cross-spectrum were determined by randomly shifting multiunit activity relative to song amplitude for all renditions, averaged across 1000 trials.

For the analysis of long syllables, we first selected syllables of lengths greater than 150ms. We then performed spectral analysis on the neural activity and song amplitude from syllable onset to 50ms prior to syllable offset. This was done in order to exclude the peak in Uva activity prior to syllable onset and the dip in Uva activity prior to syllable offset. The power spectrums were normalized by the sum of the power spectrum. To calculate the null distribution of the neural power spectrum, Uva activity was randomly scrambled then smoothed with a 2.5ms (SD) Gaussian function.

Results

Uva is necessary for adult stereotyped song

To examine the role of Uva in song production, we performed bilateral lesions of Uva in adult male birds (n=7) and found dramatic effects on song, consistent with previous reports (Williams and Vicario 1993; M J Coleman and Vu 2005). Electrolytic lesions were carried out after first mapping Uva by antidromic stimulation from HVC, and were confirmed by subsequent histology (Figure 2A). Only birds with lesions greater than 90% were considered for further analysis. Pre-lesion and post-lesion vocalizations were recorded both in social isolation (undirected song) and during the presentation of a female bird (directed song). Consistent with previously published results, we found that Uva-lesioned birds were unable to sing directed song (M J Coleman and Vu 2005). When presented with a female, lesioned birds demonstrated typical courtship behaviors, including approach and bill wiping (Zann 1996). However, these lesioned birds failed to sing and only produced sporadic short sounds, acoustically similar to introductory notes but without their characteristic rhythmicity.

While Uva lesions completely abolished directed singing, lesioned birds still sang in social isolation at rates similar to undirected singing in intact birds. However these vocalizations exhibited highly abnormal acoustic and temporal structure (Figure 2B). Visual inspection of song spectrograms revealed no apparent shared elements between pre- and post-lesion song. Furthermore, post-lesion song had no identifiable motif, and did not appear to contain syllables of reliable acoustic or temporal structure. The stereotypy of pre- and post-lesion song was quantified using a correlation-based metric referred to as the maturity index. As expected based on visual inspection of the pre- and post-lesion song, Uva lesions caused a significant decrease in the maturity index of song ($M_{pre} = 0.26 \pm 0.03$, $M_{post} = 0.08 \pm 0.02$; $p < 0.001$ paired t-test, n=7 lesioned birds, see Methods) (Figure 2G).

The loss of song stereotypy was similar to that previously reported for bilateral HVC lesions (Aronov, Andalman, and Fee 2008; Aronov et al. 2011; Veit, Aronov, and Fee 2011). Like bilateral Uva lesions, bilateral lesions of HVC profoundly alter adult singing behavior, but have differential effects on directed and undirected song. First, HVC lesions completely abolish directed song. Specifically, when presented with a female, HVC-lesioned birds approach the

female and appear to attempt singing, but no vocalizations are produced (Nottebohm, Stokes, and Leonard 1976; Aronov, Andalman, and Fee 2008). In contrast, birds with HVC lesions can still produce song in social isolation (undirected song), but their vocalizations lack the stereotyped structure of normal adult song. In fact, the undirected song produced by HVC-lesioned birds is highly variable and resembles subsong (Aronov, Andalman, and Fee 2008; Marler 1981; Veit, Aronov, and Fee 2011), the most juvenile ‘babbling’ form of singing.

Uva lesions also had a characteristic effect on the duration of syllables and gaps in these undirected vocalizations. Intact adult song contains several distinct syllables that form multiple narrow peaks in the distributions of syllable durations. In contrast, Uva-lesioned song exhibited a broad distribution of syllable durations, including an increased incidence of very long and very short syllables compared to pre-lesion song. (Figure 2C). The syllable duration distributions of Uva-lesioned birds resembled the broad exponential distribution previously described for subsong birds (Veit, Aronov, and Fee 2011). The extent to which these distributions deviated from exponential was quantified using Lilliefors statistic (Lilliefors 1969) [see **Methods**]. Post-lesion syllable duration distributions were significantly closer to exponential ($\Gamma_{\text{post}}=3.7\pm1.2$) than were pre-lesion songs ($\Gamma_{\text{pre}}=16\pm6$) ($p<0.01$ paired t-test, $n=7$ lesioned birds) (Figure 1F).

In the majority of Uva-lesioned birds ($n=6/7$), a small peak was seen in the syllable distribution between 50-100 ms that is not observed in subsong (Figure 2C). The observed deviation in syllable duration distribution may be due to the preservation of synaptically-connected neuronal chains in HVC, which could be minimally active in the absence of inputs from Uva. Another possibility is that other inputs to HVC, either Nif, Av or MMAN, may weakly activate the premotor nucleus in the absence of Uva and entrain some rhythmicity to the post-lesion song. Overall, unlike bilateral HVC lesions, Uva lesions appear to preserve some elements of song stereotypy.

Uva lesions also had a dramatic effect on the silent intervals (gaps) between syllables. Intact adult song contains gaps of discrete durations, forming multiple narrow peaks in the gap duration distribution. Following Uva lesions, however, gap durations became more broadly distributed, with an increased incidence of long and short gaps (Figure 2D). Indeed, the

distribution of gap durations in Uva-lesioned birds strongly resembled that of subsong birds (Aronov et al. 2011; Veit, Aronov, and Fee 2011), including the apparent presence of protogaps in the range of 60ms duration.

Next, we analyzed song rhythmicity, computed as the power spectrum of sound amplitude during singing. It has been shown that, during development, as vocalizations become more stereotyped zebra finch song acquires more rhythmic temporal structure (Tchernichovski et al. 2001). Given the loss of stereotypy in song of Uva-lesioned birds, we expected the power spectrum of post-lesion song to exhibit an exponential distribution similar to that seen in subsong birds. Indeed, the increase in variability in both syllable and gap durations following Uva lesions was accompanied by a dramatic decrease in rhythmicity of song temporal structure in post-lesion birds (Figure 2E). However, unlike subsong birds, Uva lesioned birds exhibited a peak in the power spectrum distribution between 3-8Hz.

We considered the possibility that the effect of lesions targeted to Uva was due to damage in surrounding thalamic tissue. Since unintended damage to surrounding tissue was largely restricted to regions dorsal or ventral to Uva, we could directly test this possibility by targeting lesions to these areas outside Uva. In two control birds, the Uva-lesion protocol was carried exactly as for experimental birds, but the lesion was targeted 250um more dorsal. In two birds, the lesion was targeted 200um more ventral. We found that, in all cases, these control lesions had no effect on song structure as assessed in song spectrograms, nor did they have a significant effect on syllable or gap duration distributions ($p=0.83$ for all measurements, $n=4$ birds) or on maturity index ($p=0.38$).

Song-related activity in Uva

Our lesion results demonstrate that Uva is necessary for stereotyped, adult song. To elucidate the nature of Uva activity during singing, we recorded from this thalamic nucleus in freely behaving adult zebra finches ($n=6$). We targeted recording electrodes to Uva using antidromic activation from HVC (Figure 3A). Single-unit recordings of antidromically-identified neurons could be obtained in anesthetized or awake non-singing birds (Figure 3B), but only multiunit signals could be recorded during singing. This was likely due to a large increase in Uva

firing rates during singing that prevented single-unit isolation. Antidromic responses had short latencies (1.5-5ms) with a small jitter ($<100\mu\text{s}$), and could be elicited by stimulation intensity of 70-300 μA (Figure 3C). Single-unit recordings of HVC-projecting Uva neurons during non-singing revealed regular spontaneous spiking at 20-50Hz ($n=4$ neurons, $n=2$ birds). Single-units recorded just dorsal or ventral to HVC-projecting core of Uva appeared to exhibit much lower rates of spontaneous spiking ($n=5$, $<10\text{Hz}$).

Multiunit activity in Uva exhibited strong modulation related to vocalizations. The multiunit signal was quantified by first rectifying and then smoothing the raw microelectrode signal (see Methods). As previously reported (Williams and Vicario 1993), Uva activity increased sharply immediately prior to the onset of distance calls (Figure 4C; latency from baseline = $23\pm6\text{ms}$, latency from peak = $9\pm7\text{ms}$). During bouts of singing, Uva activity was persistently elevated and was strongly modulated in a manner locked to song (Figure 4A). One of the most prominent features of Uva activity during singing was the robust activation prior to introductory notes (Figure 4B, latency from baseline = $39\pm8\text{ms}$, peak latency = $21\pm9\text{ms}$). Uva activity also showed a significant pattern of modulation locked to song syllables within the motif and these modulations were consistent across repetitions of the song motif (Figure 5A-C); the smoothed multiunit signals from different song renditions were highly correlated (0.64 ± 0.06 with 5ms smoothing) and coherent ($C_{\text{avg}}=0.56\pm0.16$ between 1-20Hz).

To examine the spatial homogeneity of multiunit activity within Uva, recordings were made sequentially at different depths along the same penetration. We found that activity at different recording sites throughout Uva was also highly correlated (0.62 ± 0.03 with 5ms smoothing) and coherent ($C_{\text{avg}}=0.55\pm0.17$ between 1-20Hz) (Figure 5D-F). We found no evidence that Uva activity varied depending on recording site.

We did not find evidence for modulation in Uva activity related specifically to song motifs; however, we found that Uva activity was transiently suppressed at bout offsets. Song bout offset was followed by a 200ms period of depressed neural activity in Uva relative to non-singing baseline (Figure 4A and Figure 5A).

Multiunit activity in Uva was strongly related to syllable patterning (Figure 6A), exhibiting a significant increase in activity prior to syllable onsets ($p < 0.001$ paired t-test), and a significant decrease prior to syllable offsets (Figure 6B). Uva activity peaked 15 ± 12 ms prior to syllable onset, and rose significantly above the average level of activity during singing 32 ± 12 ms before syllable onset (Figure 6C). The decrease in Uva activity at syllable offsets reached a minimum 18 ± 13 ms prior to the offset (Figure 6D) and dropped significantly below average Uva activity during singing 48 ± 17 ms prior to the offset (see Methods). These findings suggest that Uva activity is strongly related to syllable onsets and offsets. In further support of this observation, we observed a highly significant peak in the cross-correlation between Uva activity and song amplitude (magnitude of peak correlation: 0.40 ± 0.04), with a phase shift of 42 ± 6 ms (Figure 7A, \pm S.D).

Altogether, these findings suggest that modulations in Uva activity precede modulations in song amplitude with a premotor latency in the range of 15 ms to 40 ms. The chain model predicts that Uva activity exerts a premotor influence on HVC and influences the timing of song structure, such as syllable onsets. To see if our data are consistent with this view, we adopted a measure how Uva activity co-varies with these events (Ali et al. 2013). The cross-trial correlation between song-aligned time-warped spike trains across different song renditions was computed as a function of the delay of the alignment windows. For Uva multiunit activity, we observed a clear and unique maximum in this correlation at a premotor delay of 30 ± 3 ms (Figure 7B, \pm S.E, see Experimental Procedures). Based on similar analyses carried out on multiunit and single unit recordings, a premotor latency of 25-35 ms has been calculated for HVC (Lynch et al. 2016; Ali et al. 2013); thus are results are consistent with the idea that Uva activates HVC during song.

While most adult zebra finch song is not highly rhythmic, it has been reported that these songs can contain an underlying rhythm in the 10 Hz range (Saar and Mitra 2008). Indeed, we found that the song amplitude profile of our adult birds exhibited a broad spectral peak around 10 Hz (Figure 7C). Notably, Uva multiunit activity also exhibit peaks at the same frequency. Further analysis revealed a large peak in the cross spectral density between multiunit activity and song amplitude, as well as a significant coherency at this frequency ($C_{\text{avg}} = 0.58 \pm 0.13$ between 1-

20Hz, $F_{\text{peak}} = 8.8\text{Hz}$, $C_{\text{peak}}=0.75$, $p<0.01$,) (Figure 7D-E). The pronounced coherence at approximately 10Hz suggests that rhythmic modulations in song amplitude are strongly correlated with modulations in Uva activity.

Uva exhibits significant syllable-related rhythmic modulations at ~10Hz which extends through long, multi-part syllables. In these complex syllables, we observe multiple peaks in Uva activity that are consistent with the 10Hz oscillation observed in Uva. Complex syllables also exhibit multiple acoustic transitions (Figure 8A). We sought to determine whether peaks in Uva activity were associated with acoustic transitions in long, multi-part syllables. In some syllables, we did observe peaks prior to acoustic transitions (Figure 8B). However, overall we did not observe a consistent association between acoustic transitions and Uva activity; that is, we did not observe a peak in Uva preceding each acoustic transition (Figure 8C).

While Uva activity across birds reliably exhibited a 10Hz rhythm, in some syllables we also observed a striking modulation in Uva activity at frequencies higher than 10 Hz (Figure 9A). Such rapid modulations occurred over a range of frequencies, and while they were pronounced in some syllables, they were entirely absent in other syllables. To further quantify this phenomenon, we performed spectral analysis on $n=13$ individual syllables (focusing on syllables longer than 150ms in duration in order to provide adequate duration of signal). Peaks in the spectrum ranged from 12 to 55Hz. Averaged over all analyzed syllables, we found the power spectrum of Uva activity within song syllables to exhibit a broad peak between 20Hz and 50Hz ($n=8$ syllables) (Figure 9B). On average, we observed that Uva activity exhibited a significant peak in the power spectrum at ~25Hz (Figure 9C). In addition, Uva activity and sound amplitude were found to be significantly coherent across a broad range of frequencies (1-55Hz) (Figure 9D & E). This result suggests that the strong correlation between Uva activity and song amplitude is not solely due to changes in Uva activity at syllable onsets and offsets; Uva activity also appears to strongly correlated to variations in song amplitude within syllables.

Our recordings in Uva allow us to address the GTE model, in which HVC receives an efference copy of vocal-respiratory motor output initiated in the brainstem (Alonso et al. 2015). In this view, GTE-related bursts in HVC are driven by Uva, presumably by bursting activity

immediately ($<10\text{ms}$) prior to GTEs. Using a previously published automated method to identify GTEs (Boari et al. 2015) (Figure 10A), we identified GTE times in the song and calculated the cross correlation between GTE times and Uva multiunit activity. No significant peaks were observed in this correlation within individual birds or averaged across birds (Figure 10B-C).

Discussion

Using a combination of behavioral and electrophysiological studies, we examined the role of Uva in the production of stereotyped, adult song. Complete bilateral lesions of Uva result in a loss of stereotyped acoustic and temporal structure, similar to earlier reports of the effects of HVC lesions [refs]. Notably, Uva lesions resulted in a broad, nearly exponential distribution of syllable durations, characteristic of early vocal babbling [ref]. Using a motorized microdrive, we recorded multiunit activity in Uva during singing in adult birds. These recordings revealed that neural activity in Uva is strongly correlated with syllable structure, exhibiting a peak in activity prior to syllable onset with a latency of 30-42ms, and a dip in activity prior to syllable offsets with the same latency. In the frequency domain, these modulations are coherent with a pronounced 10Hz rhythmicity in song structure. Overall, while Uva exhibited syllable-related activity, we find no evidence for a specific representation in Uva of other aspects of the song hierarchy, including song motifs or song bouts.

Uva and motor representations of song

Recent efforts to relate the sparse bursting of HVC projection neurons to song structure (Amador et al. 2013) have led to the proposal that HVC bursts occur only at discrete times in the song corresponding to the onsets, offsets, and extrema of ‘gestures’ in the vocal control parameters (referred to collectively as Gesture Trajectory Extrema, or GTEs). In this model (which will be referred to as the GTE model), sub-syllabic gestures, rather than syllables, represent the fundamental unit of song production. In addition, according to the GTE model, the reported alignment between bursts and GTEs occurs with zero latency, ruling out the possibility that such bursts play a premotor role in the control of syllable onsets and offsets. In a further elaboration of the GTE hypotheses, HVC receives an efference copy, transmitted through Uva, of vocal-respiratory motor output initiated in the brainstem (Alonso et al. 2015; Amador et al. 2013). In this case, the midbrain-thalamic feedback loop should also exhibit sparse activity, locked to sub-syllabic gestures. Although several recent studies have failed to replicate the finding that HVC bursts occur with significant clustering around GTEs, and support the view that HVC bursts occur continuously throughout the song (Lynch et al. 2016) (Long 2016), our recordings in Uva may shed further light on the GTE hypothesis. A peak in Uva activity was consistently observed prior to syllable onsets, which may activate the peak in HVC interneuron

and HVC(X) projection neuron activity prior to syllable onsets (Lynch et al. 2016). However, the latency of both Uva and HVC activity occurs well prior to syllable onsets, by 20-30ms, which is not predicted by the GTE model. Furthermore, the GTE model would predict a peak in Uva activity prior to syllable offsets; instead our recordings reveal a pronounced dip at these times. Overall, we found no consistent relation between Uva activity and GTE times.

It has been proposed that the continuous activity in HVC is mediated by a synaptically connected chain of neurons (Long, Jin, and Fee 2010). According to this hypothesis, known as the chain model, activity could propagate through the HVC network—like a chain of falling dominoes—forming the basic clock that underlies song timing (Mauk and Buonomano 2004; Amari 1972; Abeles 1991). The chain model is supported by two lines of evidence. First, cooling of HVC, but not RA, slows the song, suggesting the dynamics controlling song timing exist entirely within HVC (Long and Fee 2008). Second, intracellular recordings found that during singing the subthreshold membrane potentials of HVC neurons are characterized by a large, rapid depolarization 5–10 ms before burst onset, inconsistent with a role for slow intracellular dynamics in sequence generation.

In the simplest form of the chain model, the entire motif could be generated by one long chain in HVC. However, several lines of evidence suggest that the motif is not encoded by a single continuous chain but rather by multiple discrete chains, potentially associated with syllables (Long, Jin, and Fee 2010; Michale S. Fee and Long 2013). These and other studies also suggest that complex, multi-part syllables may be composed of multiple chains. For example, bilateral, multiunit recordings in HVC reveal brief periods of interhemispheric synchronization related to syllable onsets as well as acoustic transitions within long, complex syllables, suggesting a modular organization of HVC at the level of syllables (Marc F Schmidt 2003). Further support for this view comes from detailed analysis of song timing showing that the durations of silent gaps between syllables are more variable than the durations of syllables (Glaze and Troyer 2006). Furthermore, flashes of light cause the interruption of syllables selectively at the ends of syllables or at acoustic transitions within complex multi-note syllables (Cynx 1990). Finally, local cooling of HVC has a different effect on respiratory patterns in syllables versus gaps (Andalman, Foerster, and Fee 2011). Overall, these results suggest that the

links between song syllables are mediated by a different mechanism than the structure within syllables.

One possibility is that each module or chain of neurons in HVC is connected to the next chain via a midbrain/thalamic feedback loop involving the thalamic nucleus Uva (Gibb, Gentner, and Abarbanel 2009). In this feedback loop model, HVC chains are activated by input from Uva prior to syllable onsets. Consistent with the hypothesis that Uva activates HVC prior to syllable onsets, we observed a premotor activity in Uva with a latency of approximately 30-42ms. This is slightly longer than the 29-35ms premotor latency from HVC to vocal output (Ali et al. 2013; Lynch et al. 2016), the difference likely accounted for by several milliseconds of orthodromic latency from Uva to HVC.

Several features of the Uva recordings are not explained by the feedback loop model. First, we observed persistent, elevated activity in Uva during song syllables. This elevated activity was notable for high frequency modulations that were significantly coherent with song amplitude. Second, we observed significant dips in Uva activity prior to syllable offsets. It is unclear what the significance is of either the persistent activity in syllables during syllables or the dips in Uva activity prior to syllable offset. It has been hypothesized that the activity in Uva is an efference copy of respiratory output from the brainstem during singing (Andalman, Foerster, and Fee 2011). The persistent activity in Uva during syllables may represent an efference copy of respiratory drive during syllables. The dips in Uva activity at syllable offsets may result from a decrease in expiratory drive that may occur prior to the initiation of an inspiratory pulse.

The rhythmic modulations in Uva activity coherent with an underlying ~10Hz rhythmicity in song syllable structure is a feature observed in other nuclei in the adult avian song circuit, including HVC. These oscillations in activity may have their origins in song development. In zebra finches, the adult song motif emerges during learning from an earlier stage of song development in which primitive ‘prototype syllables’ are rhythmically repeated at 10 Hz (Aronov, Andalman, and Fee 2008; Liu, Gardner, and Nottebohm 2004; Saar and Mitra 2008; Tchernichovski et al. 2001; Okubo et al. 2015). During this stage, HVC projector neurons also generate bursts with significant 10Hz rhythmicity locked to song syllables. It is possible that the 10Hz rhythmic activity we observe in the adult song circuit is a nonfunctional remnant of the early stages of song development. However, another possibility is that the avian song circuit is

functionally organized around a 10Hz rhythm, with multiple chains in HVC that span ~100ms period. According to this view, the fundamental ‘unit’ of song is not the syllable but one cycle of the 10Hz rhythm.

Synchronization of premotor activity

While song syllables are likely generated by multiple, synaptically-connected chain of neurons in HVC, the links between syllables are likely mediated by flexible midbrain-thalamic feedback connections (Michale S. Fee and Long 2013). These feedback connections are also the most probable means of interhemispheric interactions in the songbird (Marc F Schmidt, Ashmore, and Vu 2004). Thus, the strongest evidence for the modular organization of HVC may come from examining the role of the midbrain-thalamic feedback connections in synchronizing activity across hemispheres during singing.

The syrinx, the avian vocal organ, is a bipartite structure, with each half capable of functioning independently in sound generation (F Goller and Suthers 1996; Franz Goller and Suthers 1999; Suthers 1997). Because each half of the syrinx can be independently controlled to produce vocalizations, exquisite coordination is required between the syringeal motor commands that control each side (Goller & Suthers, 1996; J M Wild et al., 2000). However, several features of the avian song system make coordination a particularly challenging task. Unlike in the human language circuit, the avian song system is bilaterally organized and is anatomically symmetric across hemispheres (Paton, Manogue, and Nottebohm 1981). Each half of the syrinx is controlled by inputs from song nuclei on the ipsilateral side (J Martin Wild 1997), with the motor commands that control the system ultimately originating from the forebrain (Nottebohm, Kelley, and Paton 1982; Vicario 1991). To complicate matters further, the bird brain lacks a corpus callosum, preventing communication across hemispheres at the level of the forebrain (M F Schmidt and Ashmore 2008). In theory, without any mechanism to maintain coordination across hemispheres, any small perturbation in activity in one hemisphere can result in a temporal misalignment of motor commands and result in abnormal vocalizations.

The production of birdsong requires the precise coordination of HVC activity across hemispheres. Several lines of evidence suggest that the two HVCs are actively synchronized during singing. For example, unilateral perturbation of HVC activity by electrical stimulation

during singing leads to rapid readjustment in HVC activity in the contralateral hemisphere (35ms) (Vu, Schmidt, and Mazurek 1998). As previously mentioned, bilateral multiunit recordings in HVC reveal that right and left HVC exhibited brief periods of correlated activity prior to syllable onsets and at some acoustic transitions within longer, complex syllables (Marc F Schmidt 2003). Activity in HVC was correlated between hemispheres independent of recording site, suggesting that all regions of HVC are globally synchronized during these short periods in the song. Finally, unilateral HVC cooling does not produce a progressive distortion of the song, but rather produces normal-sounding song motifs with an intermediate amount of stretching. This result suggests that synchronization of HVC occurs at multiple points in the song rather than at a single point prior to motif onset (Michale S. Fee and Long 2013).

It has been hypothesized that the bilateral synchronization of HVC is mediated by the midbrain-thalamic feedback loop. Indeed, our findings are consistent with a role for Uva in synchronizing the two hemispheres. In particular, we observed large peaks in Uva activity 30-42ms prior to syllable onset, which is expected if Uva synchronizes HVC activity bilaterally prior to syllable onsets. However, we cannot rule out a role for other pathways in maintaining interhemispheric synchrony. There is another pathway that could also mediate the synchronization of activity in HVC across hemispheres. In this pathway, RA projects to the thalamic nucleus DMP, which projects to MMAN which then projects to HVC. This pathway completely bypasses the brainstem vocal-respiratory network. However, lesions of MMAN in adult birds have a minor effect on singing mainly restricted to introductory notes, suggesting that MMAN is unlikely necessary in the synchronization of premotor activity in HVC (Foster and Bottjer 2001).

Unclear what role Uva plays in controlling song syntax

While our findings are broadly consistent with the idea that Uva serves to bilaterally synchronize HVC prior to syllable onsets, its role in other aspects of song production remain unclear. For example, individual HVC-projecting Uva neurons could, in principle, be highly selective for individual syllable types, perhaps controlling song syntax by selectively activating particular syllable chains in HVC. Alternatively, these neurons could be active, in a non-selective way, before every song syllable, and serve simply to synchronize the two hemispheres by

simultaneously initiating HVC sequences. Finally, it remains a possibility that Uva could simply supply the excitatory tone necessary for HVC to function, without having a role either in selecting or initiating HVC sequences. Our recordings reveal a remarkable degree of homogeneity in firing patterns at different sites within Uva, hinting perhaps of a high degree of homogeneity among Uva neurons, and thus favoring a model in which Uva does not play a role in selecting syllable types. Single unit recordings of identified HVC-projecting Uva neurons will ultimately be required in order to differentiate these different models.

Uva may receive a corollary discharge from brainstem respiratory circuits

In both humans and songbirds, vocalization requires the precise coordination of respiratory and vocal-motor centers (Riede and Goller 2010; Levelt 1993). Respiration during singing consists of a stereotyped sequence of expiratory and inspiratory pressure pulses and, in adult songbirds these changes in thoracic air sac pressure are strongly correlated with changes in vocalization (Franz and Goller 2002; Veit, Aronov, and Fee 2011; Calder 1970). Each expiratory pulse (EP) is associated with a single song syllable while each inspiratory pulse (IP) is associated with a gap between syllables. This remarkably tight coordination between respiration and vocalization has led to the suggestion that forebrain circuits, particularly the premotor nucleus HVC, play a critical role in coordinating these motor processes (J Martin Wild 1998).

It has been shown that nucleus HVC is critical in controlling the temporal structure of song. Localized cooling of HVC, but not RA, slows the temporal structure of song at all timescales (Long and Fee 2008), including the fine acoustic structure within syllables, and the duration of syllables and gaps. Given the strong influence HVC cooling had on the acoustic features of song, it is reasonable to hypothesize that HVC also exhibits strong control over respiratory patterns during singing. To determine whether HVC exerts direct moment-to-moment control over respiration during singing, *Andalman et al* (2011) recorded thoracic air sac pressure in singing adult birds while bilaterally manipulating temperature in HVC. As expected, the study found that HVC cooling increased the duration of both expiratory pulses (EPs) and inspiratory pulses (IPs). However, cooling did not act on both phases of respiration identically. While cooling uniformly stretched EPs, cooling appeared to stretch most IPs non-uniformly; cooling was found to have little effect on the initial phase of the IP and majority of the cooling-induced

stretch occurred late in the IP. Many IPs appeared to change duration by either delaying or prematurely terminating the underlying inspiratory event (Andalman, Foerster, and Fee 2011).

This observation suggests that while the expiratory phase is directly driven by the nucleus HVC, the inspiratory phase is primarily driven by autonomous respiratory networks within the brainstem (Andalman, Foerster, and Fee 2011). The results from Andalman and colleagues also suggest that the brainstem vocal-respiratory network sends feedback signals to HVC during song production. Our studies of the thalamic nucleus Uva provide some evidence for this feedback signal. A striking feature of our multiunit recordings in Uva is how strongly they correlate with song amplitude which serves as an indirect measure of thoracic air pressure (Suthers and Zollinger 2004). Given the strong correlation between Uva multiunit activity and song amplitude, it is likely that Uva activity is not being driven by auditory or somatosensory feedback but instead is driven by activity in the brainstem-vocal respiratory network. This observation, along with the fact that Uva activity is largely premotor in nature, suggests that Uva may receive an ascending corollary discharge of expiratory motor commands.

Given these observations we propose that during vocalization, HVC neurons generate a sparse sequence of activity (Hahnloser, Kozhevnikov, and Fee 2002; Leonardo and Fee 2005) that continuously drives activity in RAm, causing an EP. During the EP, RAm continuously sends an efference copy of its motor output to HVC via the midbrain-thalamic feedback loop (Gibb, Gentner, and Abarbanel 2009). However, towards the end of the chain, the expiratory drive from HVC decreases. Eventually, the expiratory drive drops below a certain threshold, allowing for the initiation of an IP by PAm. When PAm initiates an IP, it also sends a signal to HVC via Uva. This signal initiates the next chain in HVC. At the onset of the next chain, there is a large increase in activity in HVC which strongly drives activity in RAm. This leads to the termination of the IP and initiation of the EP (Figure 11).

Direct and indirect projects from Uva to HVC may contribute differently to adult song production

While Uva has known projections to two telencephalic song circuit nuclei, NIf and HVC, it is unclear what the differential role of either pathway is in song production. These two

pathways are populated by two distinct, non-overlapping classes of projection neurons in Uva. The thalamic nucleus also projects to a third telencephalic song nucleus Av, though it is unclear whether this connection represents a third class of projection neurons in Uva or if this connection is mediated by collaterals of NIf/HVC projection neurons (Akutagawa and Konishi 2010). These connections, together with the results of our behavioral and electrophysiological experiments, suggest that Uva's role in the song system is remarkably extensive given its small size. Given the multiple connections Uva forms with the telencephalon, it is unclear the extent which the activity observed in Uva is reflective of the activity of its inputs to HVC versus other 'cortical' song nuclei. Ultimately, single unit recordings of the distinct classes of Uva neurons will ultimately be required in order to determine what the role of these neuronal populations in adult song production.

Thalamus and activation of cortical motor circuits

While all neocortical areas receive thalamic inputs, the functional relationship of the thalamus to the cerebral cortex remains largely unknown. In the sensory system, the classical view of the thalamus, in particular first order thalamic nuclei, is that of a relay station which receives and transmits peripheral sensory information from the external world to the cortex (Sherman and Guillery 1996; Guillery and Sherman 2002). The same may also be true about movement information. The execution of complex motor tasks requires both the generation of movements as well as monitoring of those generated movements. Information about our movements can originate from sensory receptors, including those in muscles, and from internal representations of those movements, known as an efference copy. It is likely that most, if not all, the information the brain receives regarding self-generated movements is relayed to the cortex via the thalamus (Wurtz, Sommer, and Cavanaugh 2005). However, little is understood regarding the role of higher-order thalamic nuclei in the implementation of complex motor behaviors and the processing of motor feedback information.

Information relayed by the midbrain-thalamic feedback loop may be necessary in the initiation and sequencing of behavioral units into a single, cohesive behavior. Like the midbrain-thalamic feedback loop in songbirds, a similar ascending connection between a subcortical motor center and a premotor cortical center exists in primates. The circuit, consisting of the superior

colliculus (SC) which projects to the frontal eye field (FEF) via the mediodorsal thalamus (MD), is involved in the generation of saccadic eye movements. A combination of behavioral (Sommer and Wurtz 2004b) and electrophysiological (Sommer and Wurtz 2004a) studies suggest that this midbrain-thalamic feedback pathway in primates relays a corollary discharge of midbrain motor output that is used for coordinating sequential saccades and possibly for stabilizing vision across saccades. The avian midbrain-thalamic feedback loop may act in an analogous manner. Uva may relay an efference copy of vocal-respiratory motor output from the midbrain to the ‘cortical’ premotor song nuclei of the avian brain. This signal is likely necessary to sequentially activate syllables during singing. Overall, these findings in the primate and avian brain may be a general model for how the cortex creates an internal model of executed movements. These signals may serve multiple roles such as modifying responses to sensory input from the periphery. They may also serve as a means for the cortex to track what movements have been performed and may play a critical role in the determining the temporal structure of complex behaviors (Wurtz, Sommer, and Cavanaugh 2005).

Conclusion

Here we present evidence that Uva activates HVC during stereotyped singing, and relays an efference copy of respiratory effort back to the telencephalon. As previously reported, we find that bilateral Uva lesions abolish stereotyped adult song. However, we also find that such lesions result in subsong-like vocalizations that have no distinct identifiable syllables and have a broad, nearly exponential distribution of syllable durations. Using a motorized microdrive, we recorded multiunit activity in Uva during singing in adult birds. These recordings revealed that neural activity in Uva is strongly correlated with syllable structure, exhibiting a peak in activity prior to syllable onset at a latency of 20ms and a dip in activity prior to syllable offsets at a similar latency. In the frequency domain, these modulations are coherent with a pronounced 10Hz rhythmicity in song structure. Overall, while Uva exhibited syllable-related activity, we find no evidence for a specific representation in Uva of other aspects of the song hierarchy, including song motifs or song bouts.

In conclusion, our results show that Uva is necessary in the production of stereotyped, adult song and plays a key role in activating forebrain song nuclei during singing at syllable onsets. Uva likely occupies a strategic position that allows it to coordinate activity in several brain areas across hemispheres and relay an efference copy of expiratory effort back to the telencephalon. Although future electrophysiology and other experimental procedures are needed to provide greater insight into the role of Uva in the production of adult song, our results suggest that the thalamic nucleus plays a critical role in patterning adult vocalizations.

Summary

The generation of any complex motor behavior requires the precise sequential ordering of movements. Lashley (1951) referred to this problem of coordinating constituent actions into organized sequential patterns as the “action syntax” problem. From this idea of the “action syntax” came the theory of the hierarchical organization of behavior. According to this model, all complex behaviors can be divided into multiple, simpler behavioral elements, which themselves are built of simple actions. Complex behaviors are formed by stringing these behavioral elements together in a specific order. This modular organization of behavior leads to a key question regarding the neural basis of complex, motor behaviors: Is the modular organization of complex motor behaviors reflected in their underlying neural representation in the motor control system? Using song vocalizations in songbirds as a model for a complex motor behavior, we provide evidence suggesting that the neural representation of song is modular at the level of the syllable and that each neural module is connected to the next module via a midbrain-thalamic feedback loop involving the thalamic nucleus uvaeformis (Uva).

The generation of birdsong is controlled by a discrete set of premotor nuclei including the premotor nucleus HVC. The premotor nucleus HVC projects to the robust nucleus of the arcopallium (RA) which, in turn sends projections to the various midbrain and brainstem circuits involved in the production of bird song. Nearly all of the brainstem and midbrain circuits that receive axonal inputs from RA, in turn send a projection back to HVC through the higher-order thalamic nucleus Uvaeformis (Uva), forming an anatomical brainstem-thalamocortical loop. Using a combination of behavioral and electrophysiological studies, we examined the role of the thalamic nucleus Uvaeformis (Uva) in the production of stereotyped, adult song. Complete bilateral lesions of Uva result in a loss of stereotyped acoustic and temporal structure, similar to earlier reports of the effects of HVC lesions. Notably, Uva lesions result in a broad, nearly exponential distribution of syllable durations, characteristic of early vocal babbling. Using a motorized microdrive, we recorded multiunit activity in Uva during singing in adult birds. We find that neural activity in Uva exhibits significant 10Hz rhythmicity locked to song syllables, increasing prior to syllable onsets and decreasing prior to syllable offsets—a pattern of activity observed in HVC during adult and juvenile song. These results suggest that the avian song is

functionally organized around a 10Hz rhythm, with one cycle of the 10Hz rhythm being the fundamental ‘unit’ of song.

These results are consistent with the hypothesis that Uva relays an efference copy of brainstem motor output to HVC during singing which serves to activate HVC prior to syllable onsets and synchronize premotor activity across hemispheres. Uva likely occupies a strategic position that allows it to coordinate activity in several brain areas across hemispheres and relay an efference copy of expiratory effort back to the telencephalon. Although future electrophysiology and other experimental procedures are needed to provide greater insight into the role of Uva in the production of adult song, our results suggest that the thalamic nucleus plays a critical role in patterning adult vocalizations.

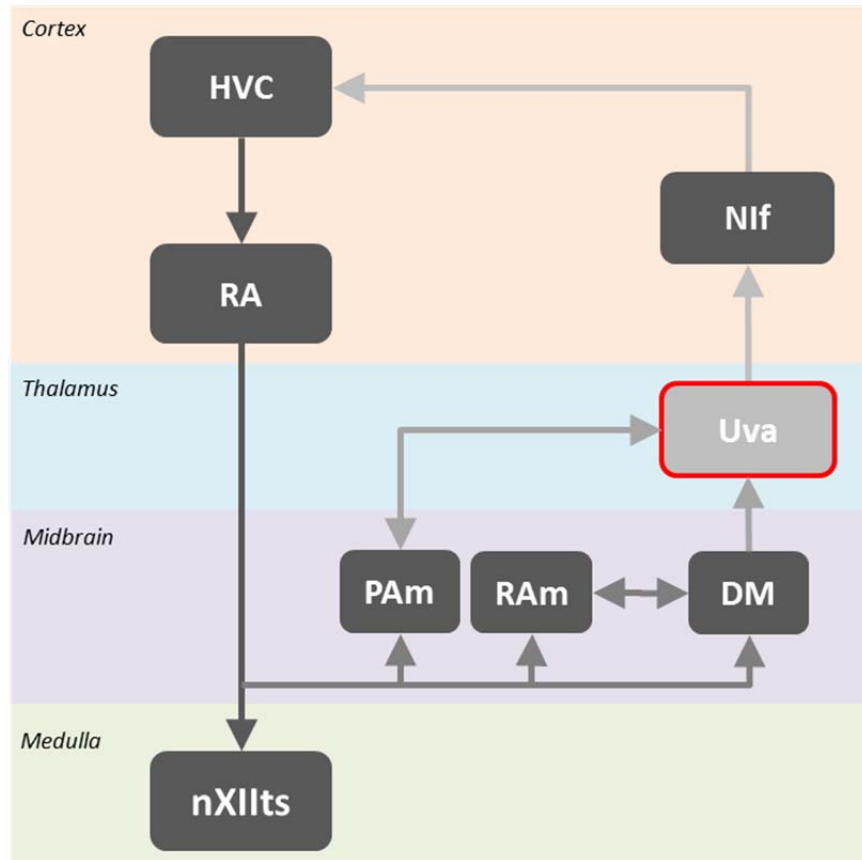


Figure 1: The avian premotor song circuit can be viewed as a combination of a feed-forward premotor pathway combined with a feedback pathway. HVC (used as proper name); RA (robust nucleus of the arcopallium); nXIIts (tracheosyringeal portion of the hypoglossal nucleus); PAm (nucleus parambigualis); RAm (nucleus retroambigualis); DM (dorsomedial medial nucleus of the intercollicular complex); Uva (nucleus Uvaeformis); Nif (nucleus interface).

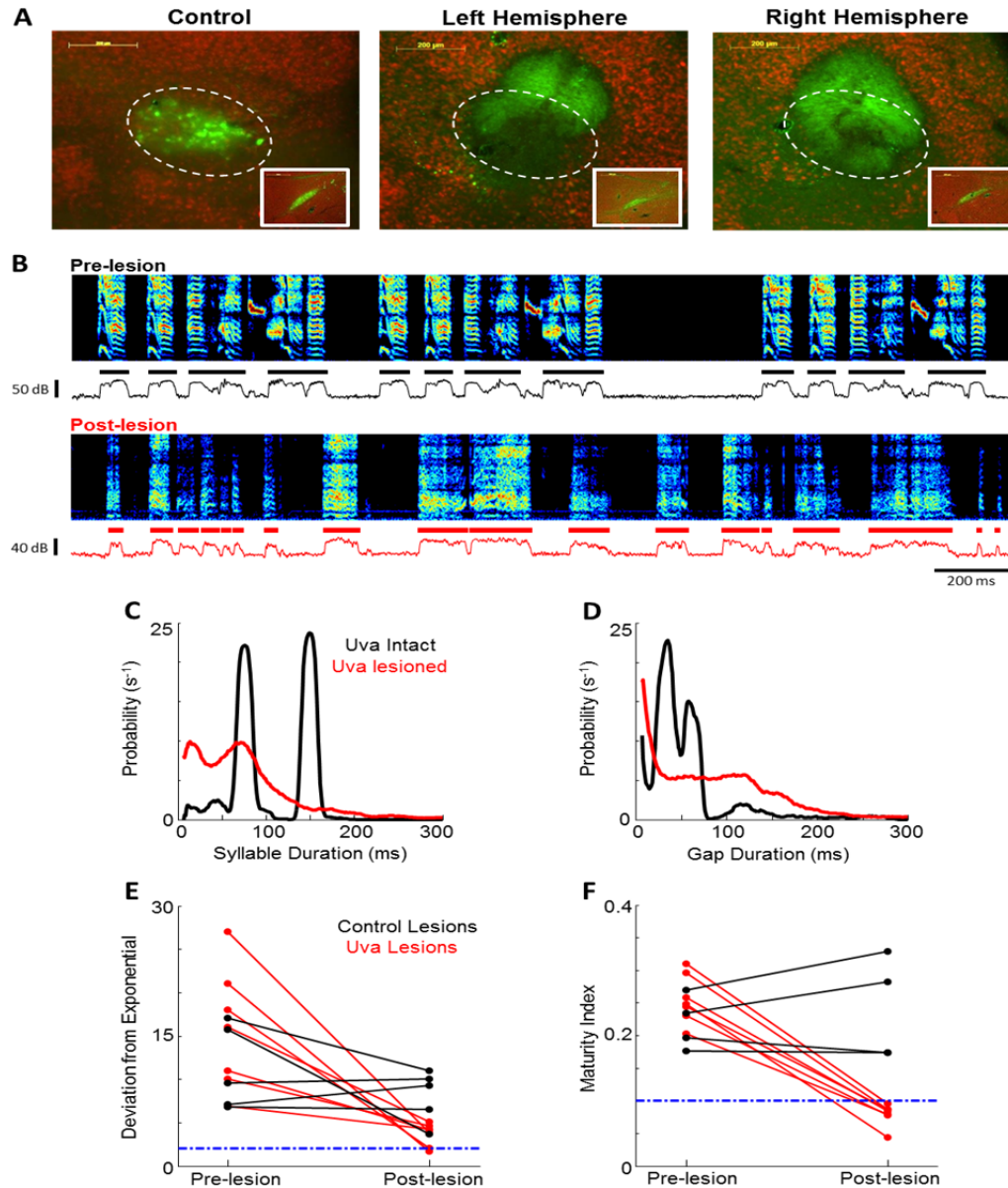


Figure 2: Song stereotypy is lost after Uva lesions. **(A)** histological verification of Uva lesions. In a control bird (left) labeled Uva-HVC projectors (green) are readily visible. Neu-N stain reveals bilateral elimination of Uva following passing of current into the center of each Uva. Dotted-line marks the border of Uva *Inset*, cells labeled in Nif from injection in HVC **(B)** (top) prelesion song spectrogram of an adult bird (>90dph). Bottom trace is the song amplitude and the black segments indicate individual syllables. (Bottom) spectrogram taken from the first day of singing after bilateral Uva lesions. Note the loss of song stereotypy in the duration of syllables and gaps and the acoustic features of the song. C and D are histograms of syllable **(C)** and gap **(D)** durations before (black trace) and after (red trace) the Uva lesions. **(E)** change in goodness of fit – a metric that quantifies how similar the syllable duration distribution is to that of subsong—in control lesion (black trace) and Uva lesioned birds (red trace). **(F)** change in maturity index in control vs Uva lesioned birds. Dotted blue line represents the cutoff for subsong.

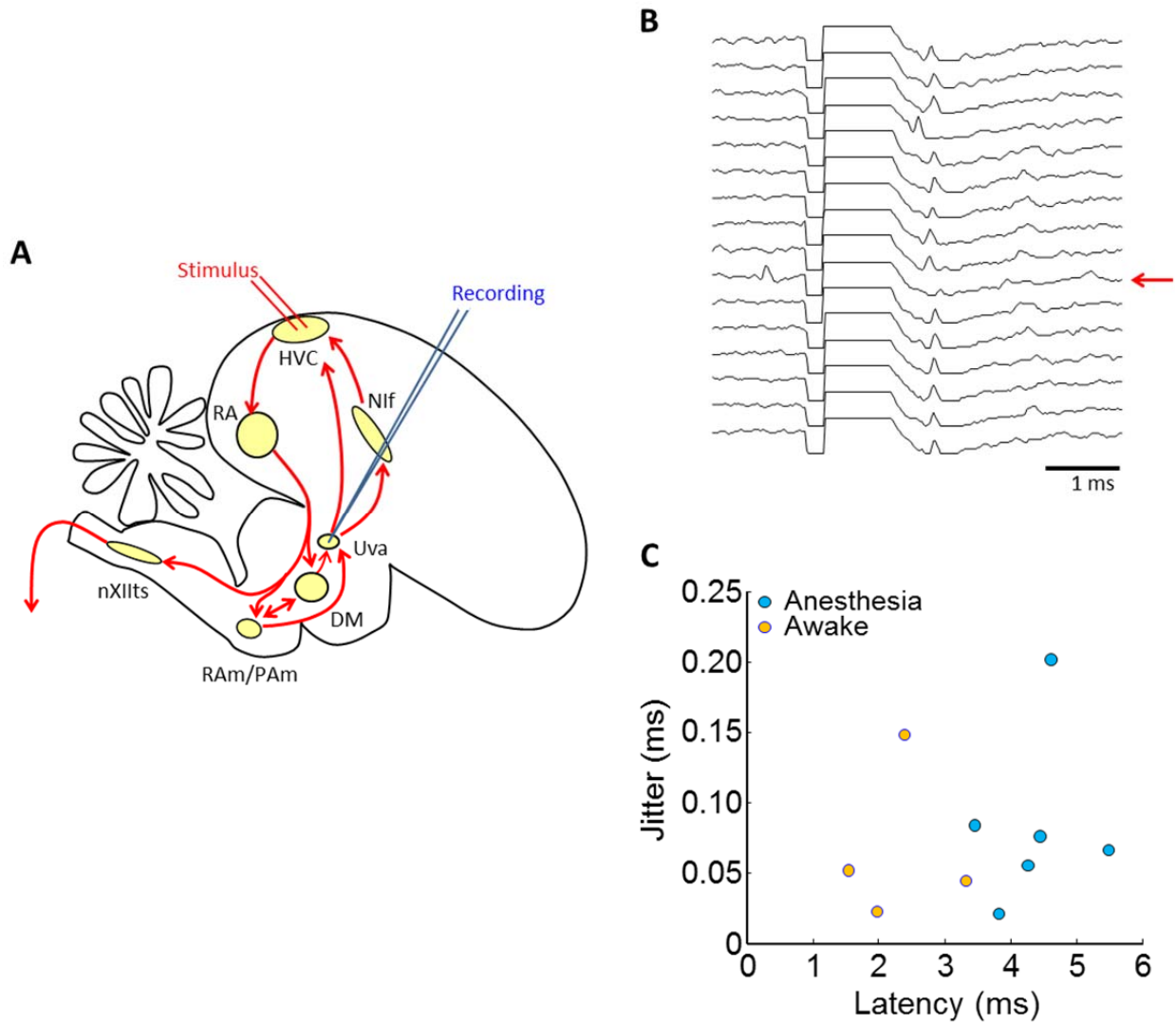


Figure 3: Recording of the activity of a large population of Uva neurons in the singing zebra finch. **(A)** Simplified schematic view of the oscine song control system. Abbreviations are HVC (proper name), RA (robust nucleus of the arcopallium), nXIIts (tracheosyringeal portion of the hypoglossal nucleus), DM (nucleus dorsalis medialis), Nif (nucleus interface) and Uva (nucleus uvaeformis). Multiunit recordings were made in Uva, which was antidromically identified by electrical stimulation in HVC. **(B)** Antidromic activation of neurons in Uva. Traces shows the response in Uva across sequential stimulations. Red arrow indicates a trial during which a spontaneous spike occurred. **(C)** Latency and jitter of antidromic responses.

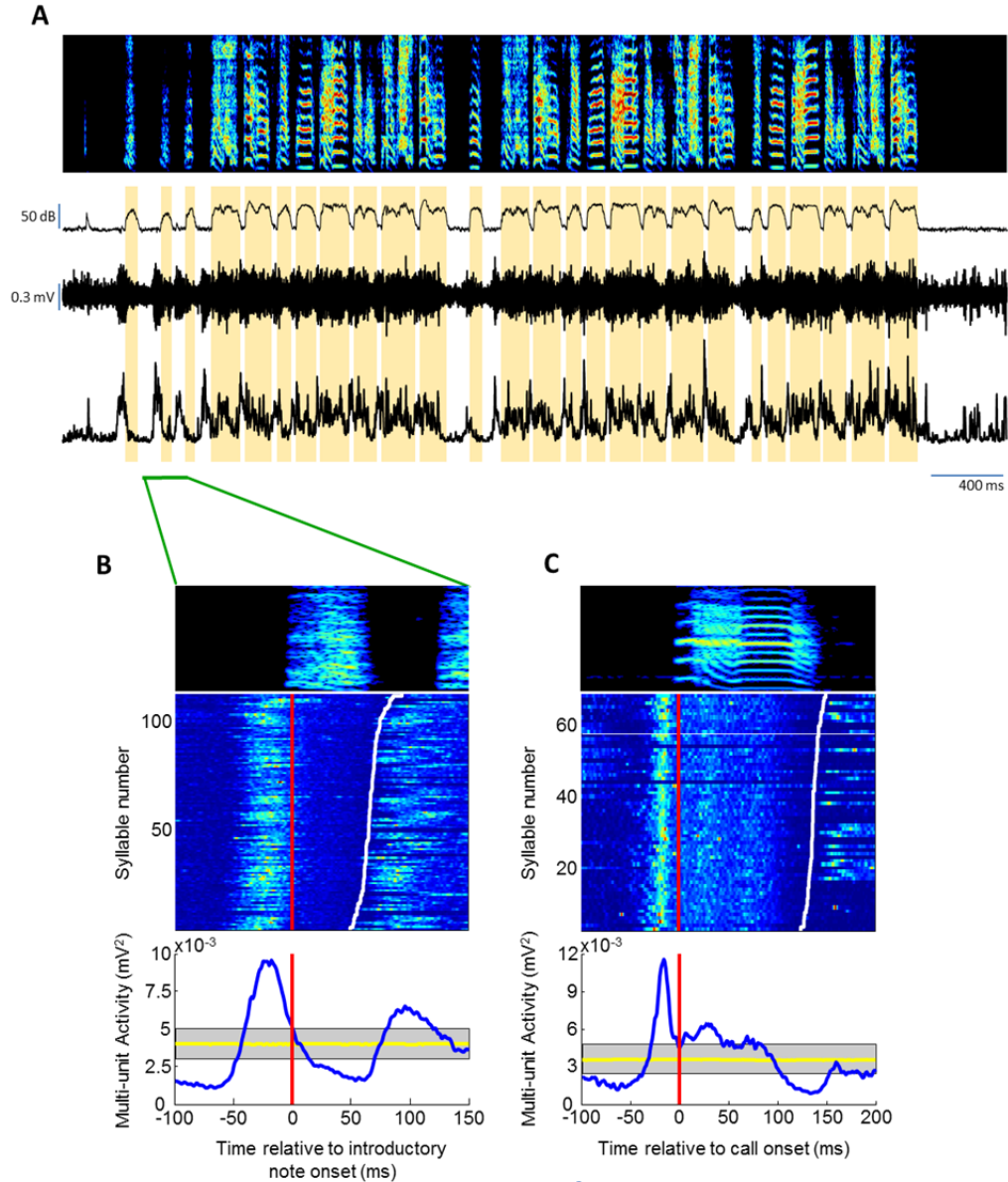


Figure 4: Premotor activity in Uva **(A)** A trace of neural activity in Uva during a single bout. song Spectrogram of an adult bird (>90dph). Trace immediately below the spectrogram is the song amplitude and the orange bars indicate individual syllables. Immediately below that is the raw neural activity followed by a smoothed and rectified neural trace. The last syllable in the song bout is followed by a period of depressed neural activity in Uva lasting for an average $200 \pm 100 \text{ ms}$ **(B)** Uva shows premotor activity prior to onset of introductory notes. At the top is a spectrogram of a single introductory note. Heat raster represents the power of neural activity during each introductory note rendition. Red line marks introductory note onset and white line marks introductory note offset. Below is a note onset aligned multiunit trace averaged across all renditions. Also shown is the 95% confidence interval of baseline activity during vocalization determined from random shuffling of multiunit activity (yellow trace). **(C)** Uva activity during calls. Uva activity peaks prior to call onset.

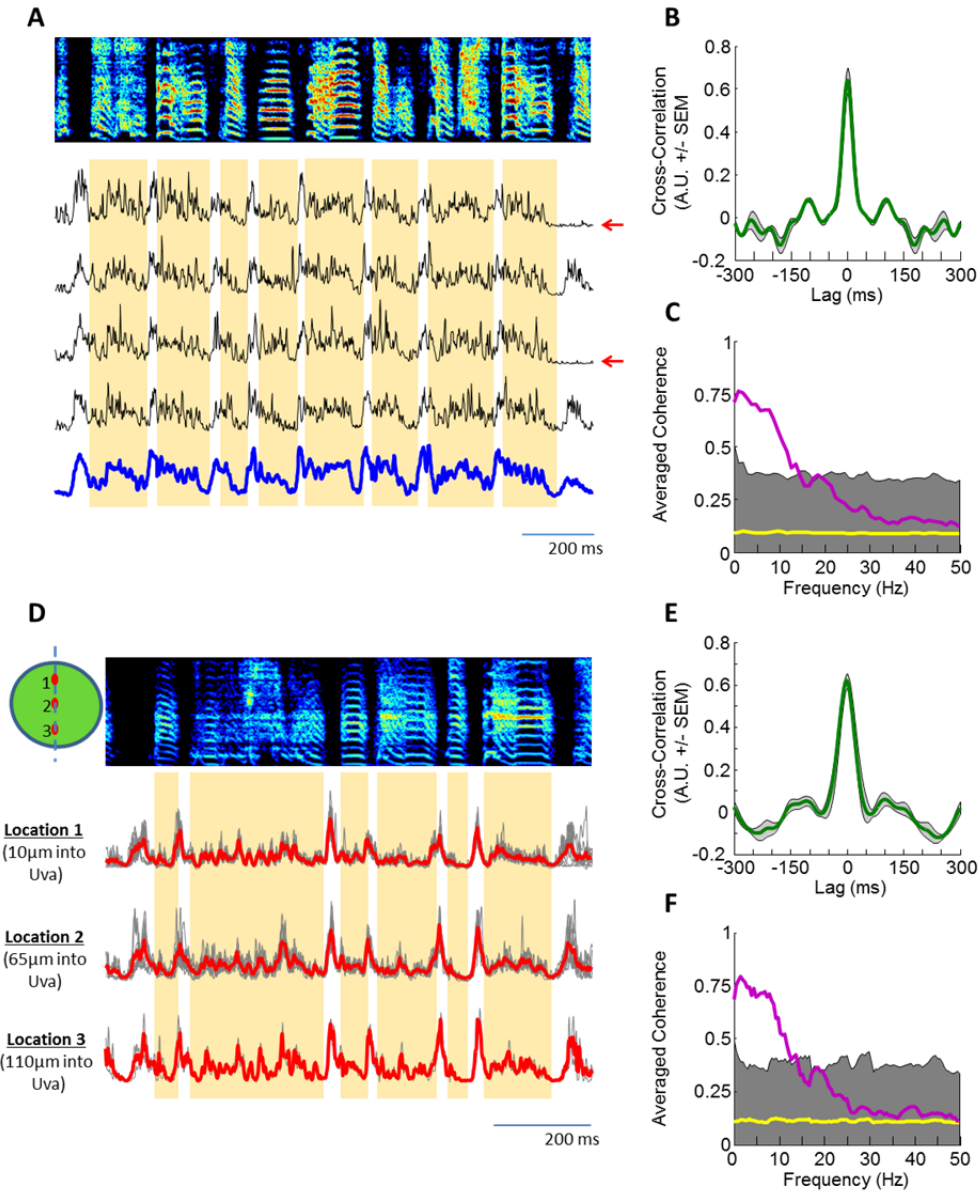


Figure 5: Uva exhibits consistent activity across multiple song renditions at multiple recording sites **(A)** Activity in Uva is consistent across multiple bouts. From top to bottom, spectrogram of a single motif, multiple traces of time-warped multiunit activity and an average trace in blue. Red arrows indicate motifs that occur at an end of a rendition. **(B)** Cross-correlation across multiple renditions, with shaded bars indicating SEM. **(C)** Coherence across multiple renditions, with shaded region indicating the 95% percentile of the null distribution corrected for multiple testing. **(D)** Activity in Uva is consistent across different recording sites. An average trace of time-warped multiunit activity at each recording site is shown in red. Individual traces are shown in gray. Syllable onset peaks and syllable offset dips are apparent. Diagram in upper left-hand corner represents the relative position of each recording site within Uva. **(E)** Cross-correlation across multiple recording sites. **(F)** Coherence measured across multiple recordings sites.

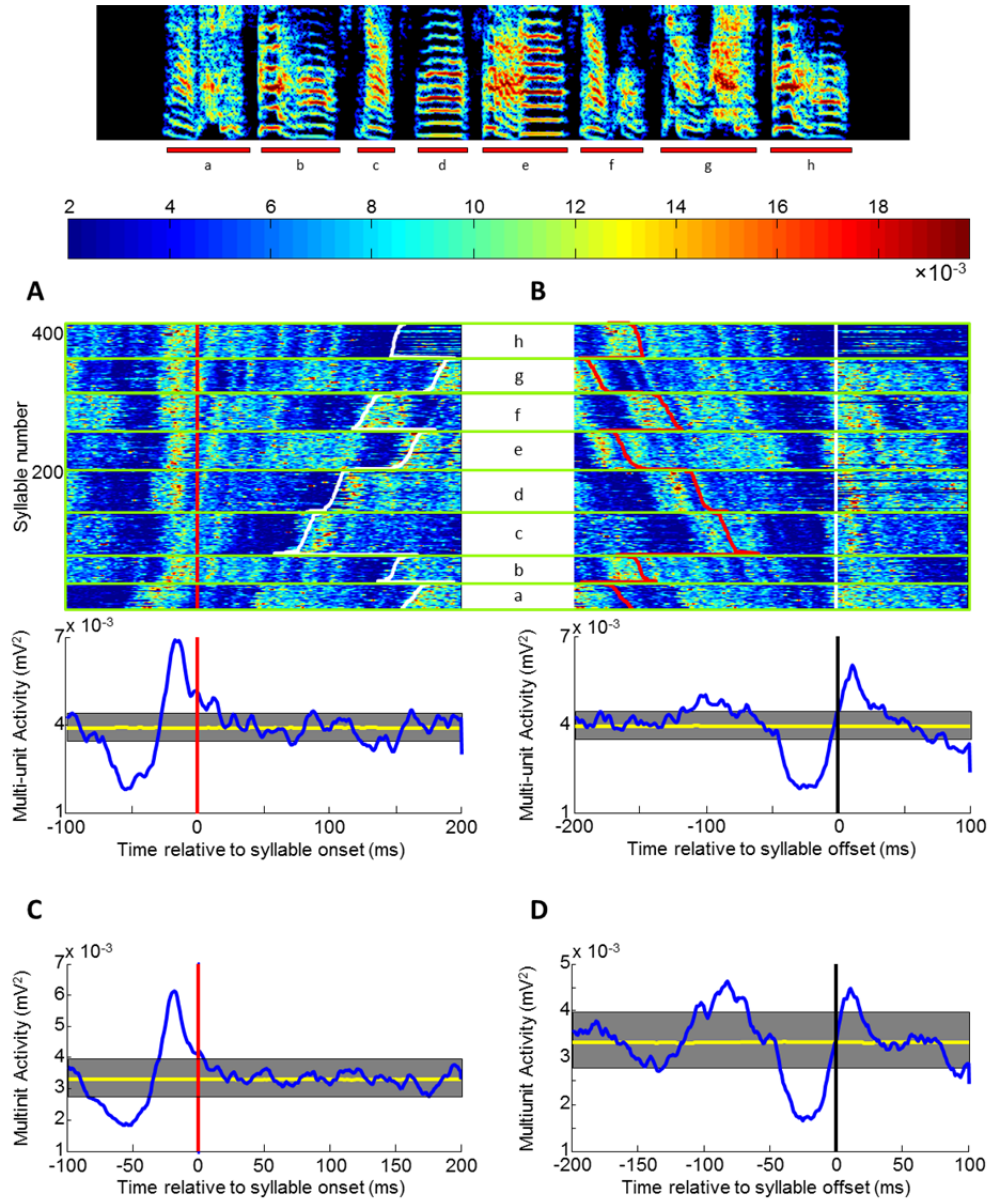


Figure 6: Uva activity peaks prior to syllable onset and dips prior to syllable offset. Above is a spectrogram of a single motif. Red bars represent the syllable lengths, with syllable labels below. **(A)** Uva activity peaks prior to syllable onset. Heat raster (top) represents the power of neural activity during each syllable rendition. Red line marks syllable onset and white line marks syllable offset. Syllables are grouped based on identity, arranged from longest to shortest syllable in descending order and then aligned to syllable onset. Individual syllables have been identified and labeled. Below is a syllable onset aligned multiunit trace averaged across all syllables. Also shown is the 95% confidence interval of baseline activity during vocalization determined from random shuffling of multiunit activity (yellow trace). Red line marks syllable onsets **(B)** Uva activity dips prior to syllable offset. Heat raster (top) shows all syllables aligned to syllable offset. Average trace (below) shows a dip prior to syllable offset. Black line represents syllable offset. **(C)** Syllable onset aligned multiunit trace across all birds. **(D)** Syllable offset aligned multiunit trace across all birds

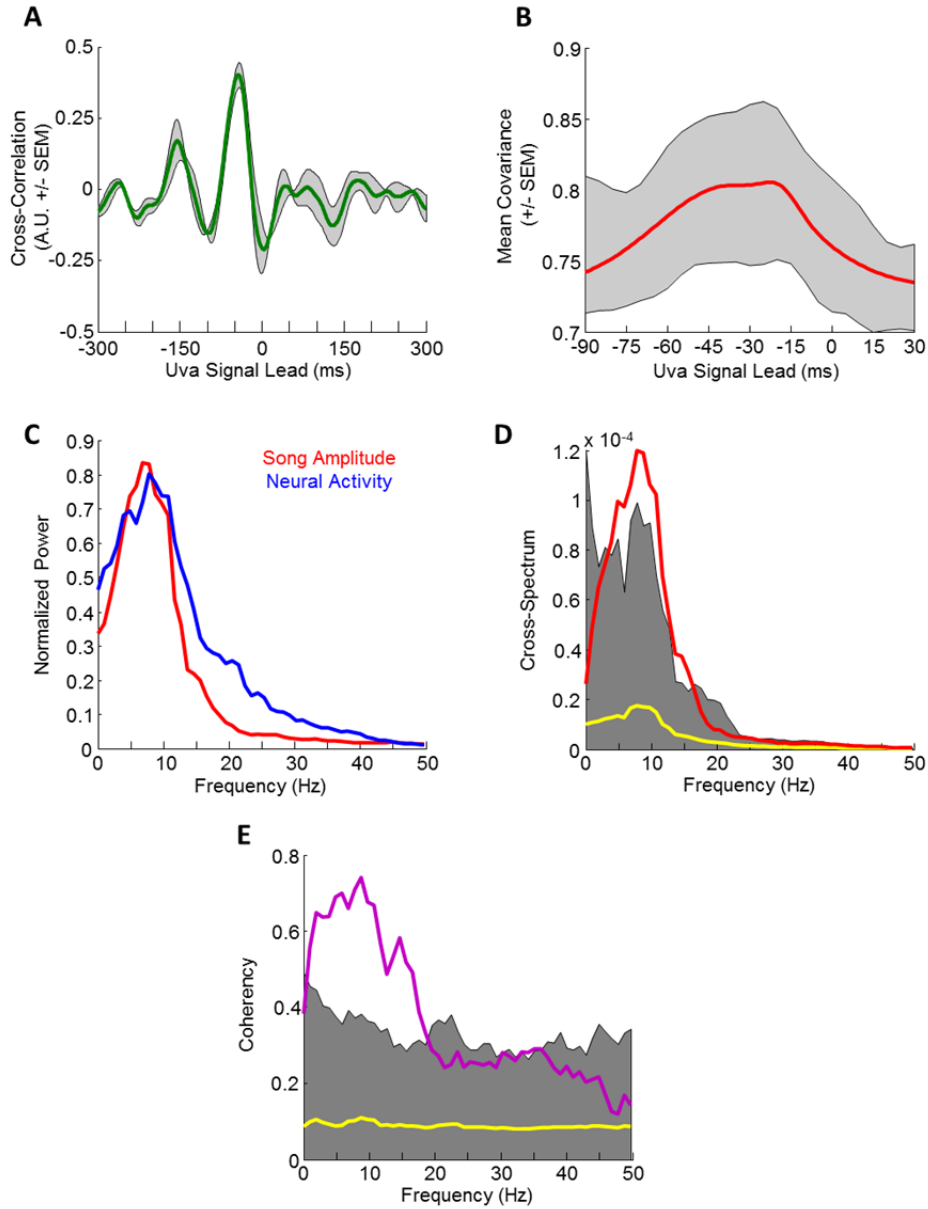


Figure 7: Quantification of rhythmic activity in Uva **(A)** Cross-correlation function between Uva activity and sound amplitude averaged across 5 birds (solid line: mean, shaded region: SEM; peak correlation = 0.40 ± 0.04 , mean lag at peak correlation = 42 ± 6 ms). **(B)** Estimated covariance between the temporal variability in Uva activity and song over a range of Uva signal lead times (see Experimental Procedures). A Gaussian fit to this data ($\mu_1 = -36.8$ ms, $\sigma_1 = 4.3$ ms, $\mu_2 = -18.3$ ms, $\sigma_2 = 2.1$ ms, $y_0 = -0.73$, $R^2 = 0.99$) is shown in red. **(C)** normalized power spectra of the song amplitudes and Uva multiunit activity, averaged across $n=5$ birds. A broad peak in the power spectrum was seen in both the song amplitude and neural data, centered around 10 Hz. **(D)** Cross-spectrum between Uva activity and sound amplitude averaged across $n=5$ birds (red line: mean, yellow line: null cross-spectrum, shaded region: 95% percentile corrected for multiple testing) **(E)** Coherency between Uva activity and sound amplitude averaged across $n=5$ birds (purple line: mean, yellow line: null cross-spectrum, shaded region: 95% percentile corrected for multiple testing). Note a large, significant peak is observed in both the cross-spectrum and the coherence at ~ 10 Hz.

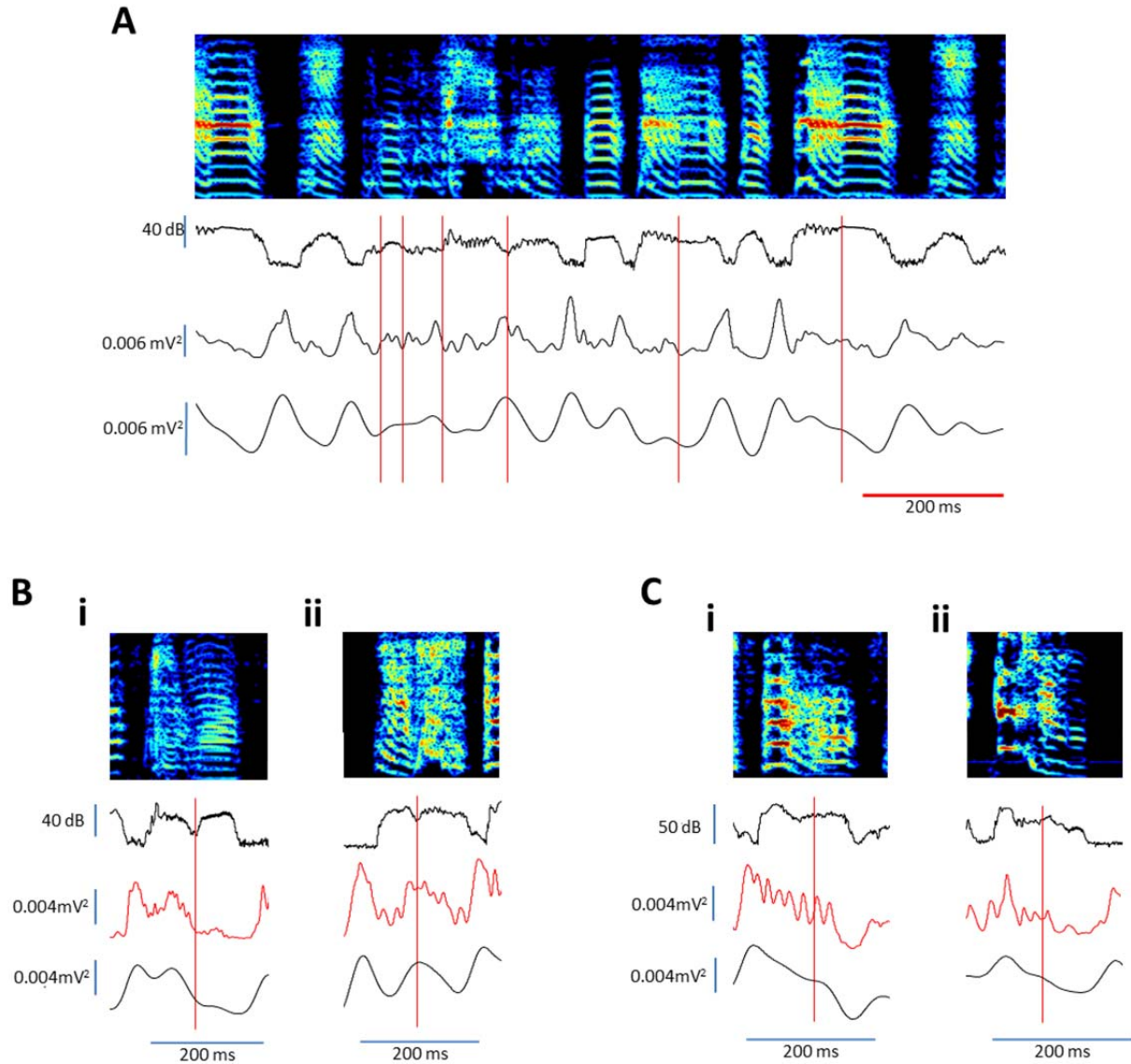


Figure 8: Uva activity exhibits significant peaks prior to acoustic transition in long syllables. **(A)** Many syllables in a song may exhibit one or more acoustic transitions. Song spectrogram of an adult bird (>90dph). Trace immediately below the spectrogram is the song amplitude. Immediately below that is the smoothed and rectified neural trace. Bottom trace is of the multiunit activity after being filtered. Red lines mark acoustic transitions within long syllables. **(B)** Examples of syllables where peaks in Uva activity are associated with acoustic transitions. **(C)** Examples of syllables where peaks in Uva activity are not associated with acoustic transitions.

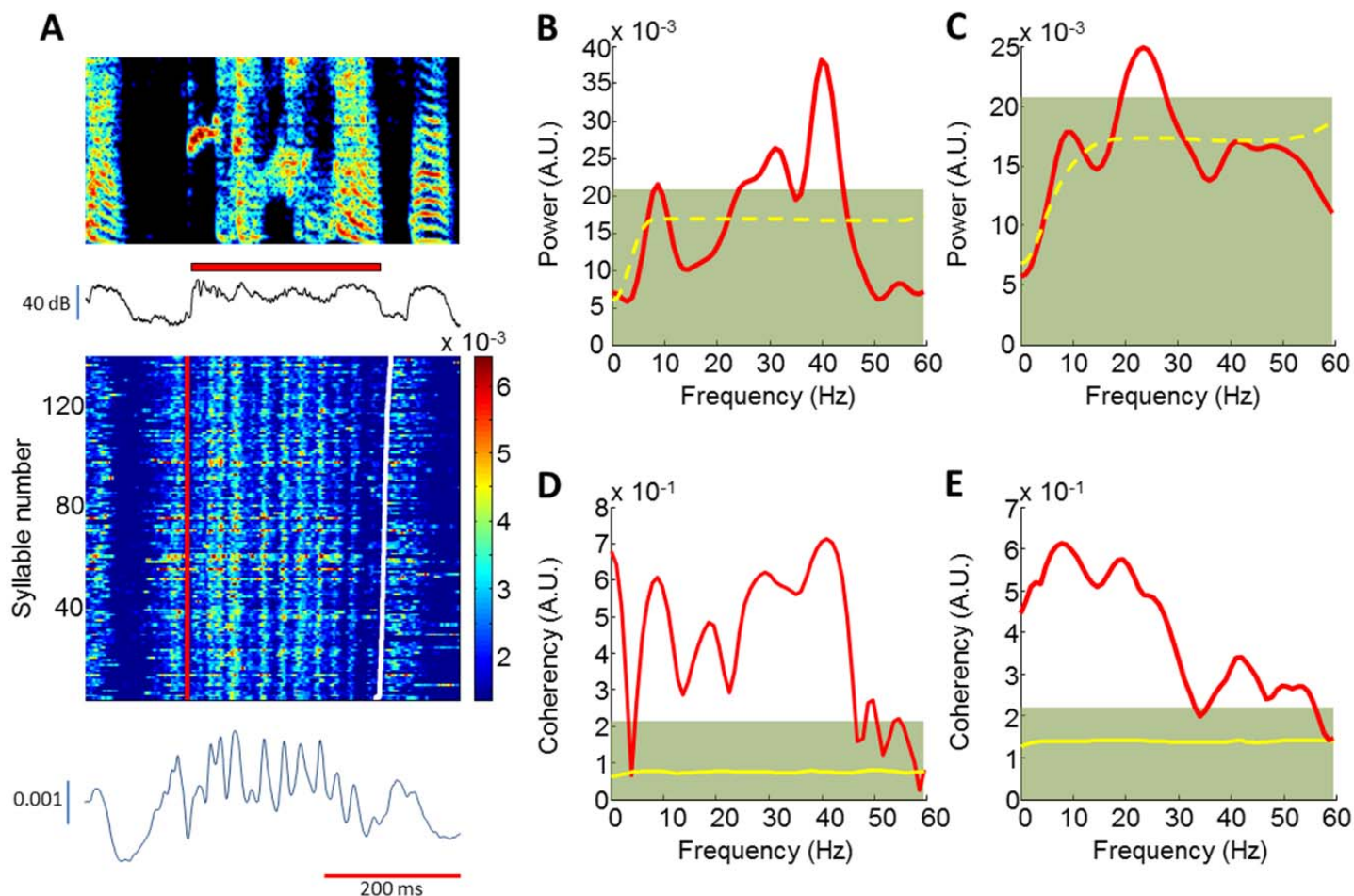


Figure 9: Rhythmicity during long syllables. (A) In many long syllables (>150ms in length), we observed rapid oscillations in Uva activity. (B) In these long syllables, we consistently observed a peak in the power spectrum of Uva activity between 20-30Hz and 40-50Hz. (C) When we averaged the power spectrum across all long syllables with a significant peak in the power spectrum >10Hz, we found that on average there was a peak in the power spectrum at ~25Hz. (D) Uva activity during these long syllables is significantly coherent with song amplitude across a large frequency range (1-55Hz) and (E) across all birds.

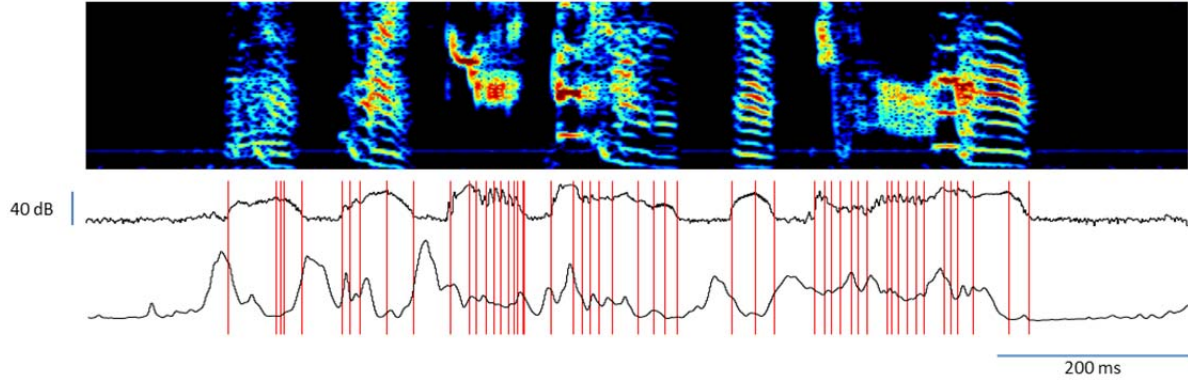
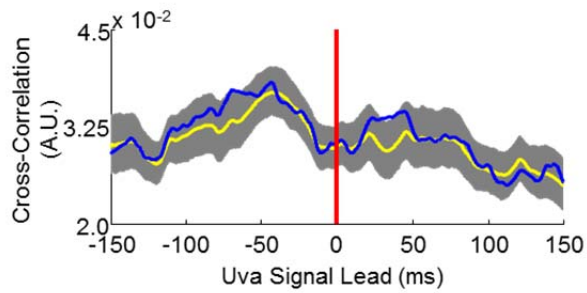
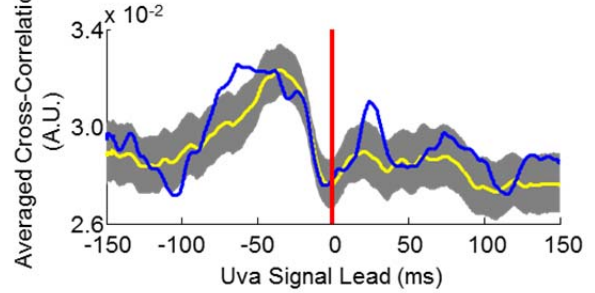
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Figure 10: Uva activity shows no significant correlation with GTE times. (A) GTE were identified using automated algorithm. (B) Uva activity does not exhibit any significant correlation with GTE times from 0-50ms prior to GTE transitions and this relationship persists averaged across (C) all birds (gray bars: 5-95% confidence interval of null distribution; yellow: null distribution; blue: cross-correlation between GTEs and Uva activity).

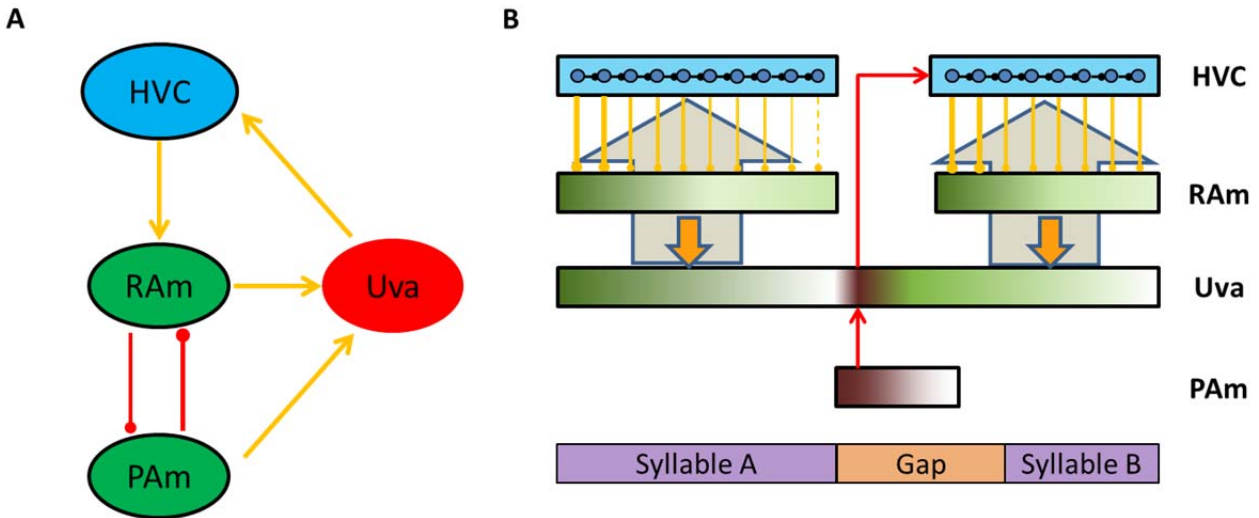


Figure 11: Hypothesis of brainstem feedback during singing. A) A simplified diagram of the avian song circuit including the premotor nucleus HVC, the respiratory network and the thalamic nucleus Uva. HVC drives activity in the respiratory nucleus RAm, which drives expiration. In the brainstem respiratory network, RAm and PAm mutually inhibit each other. Both PAm and RAm send direct and indirect connections to Uva, respectively. B) Temporal structure of song is controlled by multiple, synaptically connected chains in HVC. As a chain fires, it activates the respiratory nucleus RAm, driving an expiratory pulse (EP). During this time, RAm is continuously sending an efference copy of expiratory drive (Large gray arrow) back to HVC via the thalamic nucleus Uva (Solid orange arrow). As the chain reaches towards the end, expiratory drive from HVC decreases. After expiratory drive drops below a threshold, PAm is activated, initiating an inspiratory pulse (IP). PAm sends a signal back to HVC via Uva, initiating the next chain (red arrow). Expiratory drive from HVC rapidly increases, the IP is termination and the next EP/syllable is initiated.

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